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Soybean Peroxidase Catalysis in Removal of Anilines and Azo-Dyes from Water

by

Samar Mazloun

A Dissertation

Submitted to the Faculty of Graduate Studies

Through the Department of Civil and Environmental Engineering

In Partial Fulfillment of the Requirements for

The Degree of Doctor of Philosophy at the

University of Windsor

Windsor, Ontario, Canada

2014

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Soybean Peroxidase Catalysis in Removal of Anilines and Azo Dyes from Water

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Declaration of Originality

I hereby certify that I am the sole author of this dissertation and that no part of this dissertation has been published or submitted for publication.

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ABSTRACT

Azo-dyes are the largest group of colourants produced, and they are applied in many industries. In the environment they are recalcitrant, and under anaerobic conditions can break down to toxic or even carcinogenic aromatic amines. Aerobic treatment of azo-dye-contaminated waters has been shown to be ineffective. Thus, enzyme-catalyzed polymerization and precipitation of azo-dyes and their reduction products was studied and optimized in this dissertation. Additionally, zero-valent iron reduction of azo-dyes under anaerobic conditions followed by soybean peroxidase (SBP) enzymatic treatment was investigated. The use of additives to reduce enzyme requirement and enhance the removal of anilines was also studied. Azo-dyes and authentic anilines were treated at 1 mM, while the anilines recovered from zero-valent iron reduction were treated at 0.5 mM. All experiments were conducted in batch reactors, and the parameters: pH, hydrogen peroxide to substrate ratio, enzyme concentration and additive concentration were optimized.

Enzymatic treatment was successful in removal of 95% of both aniline and *o*-anisidine. The use of additives, sodium dodecyl sulfate (SDS), sodium dodecylbenzenesulfonate (SDBS), Triton X-100, and sodium dodecanoate (SDOD), reduced enzyme dose requirement, while the use of polyethylene glycol (PEG, average molar mass of 3350 g/mole) had no effect on the required enzyme dose. In addition, the presence of SDS also enhanced treatment by improving precipitation and removing colour.

Azo-dyes treated with SBP directly were successfully decolourized, with 85% colour removal of Acid Red 4 (AR4) and 95% for Crocein Orange G (COG). The pretreatment of AR4 with zero-valent iron, was able to achieve an even higher percent of decolourization 95%, while the second stage of treatment with SBP removed >95% of the recovered *o*-anisidine and further decolourized the water.

DEDICATION

To Mom and Dad for teaching me to reach for the stars, all while being there if I fall.

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CHAPTER 1

Introduction

Coloured water is the easiest way to identify water pollution. As little as 10 mg/L of dye can be highly visible (Yu et al., 2001). It is not only aesthetically displeasing, but can be toxic to aquatic life, and interfere with photosynthetic processes in streams (Nawar & Doma, 1989). Major contributors to coloured waters are the textile industries through their dyeing and finishing processes (Dahghani et al., 2011). However, dyes are also used in many other industries including leather, plastics, cosmetics and food processing industries (Stolz, 2001).

1.1 Dyes

Worldwide, more than 7×10^5 metric tons of synthetic dyes are produced per year, of which about 10% is discharged as effluent (Yu et al., 2001; Stolz, 2001). Dyes can be classified according to their chromophores constituting azo, anthroquinone and indigo (Yu et al., 2001). They can also fall under three classes according to their structure and charge, cationic, non-ionic and anionic. These are further sub-classified into direct, acid, and reactive dyes, which fall under anionic dyes; whereas disperse dyes are non-ionic (Robinson et al., 2001). Reactive dyes have an even higher percentage, up to 50%,

released in the dye bath effluent. These dyes react with the ionized hydroxyl groups on cellulose fibers. Due to the alkaline dyeing conditions, hydroxyl ions compete with the cellulose substrate resulting in a certain percentage of the dye unable to bind to the fiber (Carliell et al., 1996).

Among synthetic dyes, azo-dyes are considered to be the most important group (Rodriguez Couto & Toca-Herrera, 2006). These are characterized by aromatic rings joined by at least one (-N=N-) bond. Azo-dyes comprise over 50% of all the dyes, accounting for more than 2000 different compounds (Carliell et al., 1995; Carliell et al., 1996; Stolz, 2001). This group of dyes is highly recalcitrant and thus form a very important class of xenobiotics, that do not degrade readily under aerobic conditions (Pakshirajan & Singh, 2010; Stolz, 2001; Stibrova et al., 1996). However, certain aerobic bacteria are able to cleave the azo group (Stolz, 2001). Those dyes that degrade anaerobically, or undergo oxidative conversion, form intermediates, such as aromatic amines, exhibiting toxic and carcinogenic effects (Stibrova et al., 1996; Biswas et al., 2007; Rodriguez Couto & Toca – Herrera, 2006). Two representative dyes were chosen in this study, Crocein Orange G (COG) and Acid Red 4 (AR4). The reason for their selection is upon cleaving of the azo linkage they are reduced forming aromatic amines, aniline and *o*-anisidine respectively, which are considered toxic and are of regulatory concern.

1.2 Aromatic Amines

Aromatic amines are identified by one or more aromatic rings bearing amino substituents with aniline being the simplest structure. They are released in the effluent of many industries such as chemical manufacturing, coal conversion, resin and plastic

manufacturing, pesticide, pharmaceutical, explosive, textile industry as well as the dye manufacturing industry (Klibanov and Morris, 1981; Klibanov et al., 1983; Karim and Hussein, 2009). Most aromatic amines are toxic and many are classified as carcinogenic or probable human carcinogens (Kilbanov and Morris, 1981; Pinheiro et al., 2004; Karim and Husain, 2009; Casero et al., 1997). As early as the late 19th century, aromatic amines exposure has been viewed as a concern in the dye manufacturing industry (Karim and Husain, 2009). They have been shown to biodegrade, however this process seems to be more complicated than the reduction of the azo linkage. In certain cases specific microbial strains or co-cultures may be required (Pinheiro et al., 2004). Thus, a problem of bioaccumulation may arise, posing a further risk to aquatic life (Suzuki et al., 2001). Under certain circumstances, these aromatic amines can become mutagenic in mammals (Spadaro et al., 1992). However, toxicity and eco-toxicity data are lacking for many chemicals, and new ones are being manufactured, thus regulatory measures are being influenced by the precautionary principle, whose goal is to reduce chemical discharge on the assumption that they can pose a hazard (Pinheiro et al., 2004). Azo-dye linkages can possibly breakdown to aromatic amines during their use or disposal and thus are considered a health hazard (Pinheiro et al., 2004). These factors are influencing environmental regulatory agencies to develop more stringent effluent standards (Banat et al., 1996). In particular this study focuses on aniline and *o*-anisidine both are identified in U.S. EPA Toxics Release Inventory (TRI) as toxic and will be further discussed in Section 2.1.

Treatment of aromatic amines has been achieved through adsorption, extraction, microbial or chemical oxidation, electrochemical techniques, and irradiation (Slein and

Sansone, 1980). However, these processes are limited by their high cost, incomplete treatment, hazardous byproducts, and low concentration effectiveness (Husain and Jan, 2000). Chemical oxidation is the most widely used treatment, however it is dependent on the oxidant, amine structure and reaction conditions. In certain cases the byproducts can also be carcinogenic or can be reduced to the original amine by mild reductants (Casero et al., 1997). Treatment of these compounds via activated sludge systems depends highly on the nature of aromatic amine, while some could be metabolized by the system, others showed little to no reduction (Baird et al., 1977). The use of different micro-organisms has shown potential for complete mineralization. However, the disadvantages to this include high cost of microbes, which are normally substrate specific requiring additional alternate carbon sources, and metabolic inhibition (Husain and Jan, 2000).

1.3 Colour Removal Methods

Due to the complicated nature of textile and dyestuff industry effluent, conventional wastewater treatment processes do not easily remove azo-dyes (Pasti-Grigsby et al., 1992; Rodriquez Couto & Toca-Herrera, 2006). The difficulty in their treatment is attributed to their manufacture of azo-dye characteristics that are stable to sweat, soap, water, light or oxidizing agents in order to maintain their brilliant colour (Banat et al., 1996). Currently the treatment of these dyes is carried out by physiochemical methods, such as adsorption, coagulation and flocculation, chemical oxidation (ozone, hydrogen peroxide and chlorine), membrane filtration, ion exchange, photodegradation, irradiation, and electrochemical destruction (Stolz, 2001; Banat et al., 1996; Shaffiqu et al., 2002). The drawbacks to all these processes include excess chemicals requirements,

production of large quantity of sludge, disposal restrictions, production of toxins and high operating and capital costs (Banat et al., 1996; Shaffique et al., 2002).

Physical methods include adsorption techniques with adsorbents such as activated carbon, peat, wood chips, fly ash, and coal, and silica gel (Robinson, 2001). Activated carbon is inefficient in the treatment of insoluble dyes (Ahn et al., 1999). For soluble dyes, the process is effective but can be costly due to chemicals needed for regeneration of the carbon. Costs also increase due to the need for safe disposal of the concentrated and more toxic byproduct (Robinson et al., 2002). Membrane filtration though resilient to the complicated nature of dye effluent, is mostly effective with low concentration effluent. Problems of clogging, residue disposal and membrane replacement can be costly (Robinson et al., 2001). Ion exchange is considered to be a costly process and only effective on ionic dyes (Cao, 2000).

Chemical treatment by coagulation is widely used in Germany (Vandevivere et al., 1998). The process produces a large amount of sludge creating another costly disposal problem, and in certain cases leads to the need for combustion of toxic sludge (Ahn et al., 1999; Vandevivere et al., 1998). An effective chemical treatment is through oxidation by ozonation. It is able to decolorize all dyes with the exception of disperse dyes. A drawback to ozonation is its inability to remove COD in these effluents, its decolorization effectiveness is reduced by impurities leading to an increase in treatment cost, and the process might create toxic byproducts (Ahn et al., 1999; Arslan-Alaton, 2003). Photochemical processes such as UV treatment are found to be effective only on effluents with low dye concentration (Dehghani et al., 2011). Advanced oxidation processes such as UV/TiO₂ has been shown to be effective in the treatment of azo-dye at a high light

intensity and catalyst loading (Wang et al., 2008). Another promising method involved ozonation followed by UV, which removed colour as well as 90% of residual total organic carbon (Huang et al., 1994).

Activated sludge processes seem to be hindered in azo-dye treatment due to their xenobiotic nature (Coughlin et al., 1999; Yu et al., 2001). Also, some carcinogenic aromatic amines were shown to be toxic and resistant to bacterial degradation, with acclimated systems being able to at best partially metabolize many mono-aromatics (Baird et al., 1977). A combination of both anaerobic and aerobic treatment process would also yield a good outcome for dye effluent (Coughlin et al., 1999). However, a large volume of sludge that would require treatment and disposal would have to follow (Rott and Mike, 1999). On the other hand, a more effective process which has been shown to treat dyes in this manner is iron reduction under anaerobic conditions followed by enzymatic treatment (Biswas et al., 2007; Biswas et al., 2004). Enzymatic treatment is an efficient method to remove a wide range of these dyes and their aromatic amine substituents (Stolz, 2001; Pasti-Grigsby et al., 1992; Rodriguez Couto & Toca Herrera, 2006; Husain et al., 2009; Regalado et al., 2004). Advantages to the use of enzymes is their ability to react with their substrates with high specificity, without being effected by shock load as with microorganisms (Nicell, 2003). As well, they are easier to handle, store and control because of their independence of microbial growth (Mantha et al., 2002 as per Vieth and Venkatasubramanian, 1973).

1.4 Enzymes

Enzymes are classified into six main classes, oxidoreductases, transferases, hydrolases, lyases, isomerases and ligases. They are biological catalysts that increase chemical

reaction rates (Palmer, 1991), thus promoting oxidation of many aromatic compounds. Their application in waste treatment was first proposed in the 1930s (Atiken, 1993). However it was not until the 1970's when they were first used to degrade specific pollutants such as pesticide organophosphates (Munnecke, 1976). In 1980 Klibanov et al. developed a new enzymatic method for the removal of aromatic pollutants from waste waters by peroxidases.

Enzymatic treatment of aromatic amines and azo-dyes has been claimed or proposed as a preferential method of treatment over conventional treatment with microorganisms (Nicell, 2003). In conventional biological treatment microorganisms produce enzymes, which then degrade chemical compounds; however there, is the potential for the inhibition of the microorganisms before the enzymes are produced (Nicell, 2003). Isolated enzymes can act with greater specificity, are independent to bacterial growth and thus do not need acclimatizing time, can handle shock loads more readily and are simpler to control (Taylor et al., 1996). In conventional treatment the target compounds are broken down, where as in enzymatic removal they are built up through the process of polymerization (Saha et al., 2008). This allows for precipitation of organics and their removal by sedimentation or filtration. Overall, enzymatic treatment is viewed as a low energy and chemical consumption process (Demarche et al., 2012).

Enzymatic treatment does have its drawbacks. One of the major disadvantages of enzymatic treatment is the cost of the enzyme. However it should be noted that as of 2009 the enzyme world market was worth \$5.1 billion, a figure that has grown significantly from the \$1.6 billion in 1998 (Sanchez and Demian, 2011). New biotechnological advances in genetic manipulation of microbial and plant cells and

improved efficiency of isolation and purification procedures are paving the way for cheaper production of enzymes (Nicell, 2003). Another limitation to enzymatic treatment is the possible enzyme inactivation during the treatment process. This may result in the need for an increased amount of enzyme to achieve total treatment of pollutants (Wu et al., 1993). Additives have been used successfully by Nakamoto and Machida (1992) in significantly reducing the amount of enzyme needed. A wide variety of azo-dyes have been treated using a number of different enzymes, such as, horseradish peroxidase (Stibrova et al., 1996; Bhunia et al., 2001; Mohan et al., 2005; Ulson de Souza et al., 2007), *Pseudomonas* reductases (Yu et al., 2001), lignin peroxidase (Ollikka et al., 1993) *Ipomea* & *Sacharum* peroxidases (Shaffique et al., 2002) *Arthromyces ramosus* peroxidase (Biswas et al., 2007). In particular 50 μ M of COG and AR4 were treated with *Arthromyces ramosus* peroxidase by Wang (2002).

The wastewater problem of azo dyes and toxic anilines is not just limited to the dye industry and regulations have been put in place to monitor and limit their use and release. Thus a viable treatment method is needed to resolve their effluent colour and toxicity. During this study 1 mM of COG and AR4 along with their aniline reductive products were treated with soybean peroxidase.

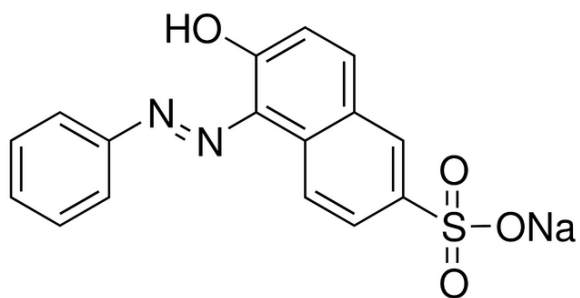
1.5 Objective

- To optimize soybean peroxidase (SBP: EC 1.11.1.7) catalyzed removal of two representative azo-dyes Crocein Orange G (COG: CAS No. 1934-20-9) also known as Acid Orange 12 and Acid Red 4 (AR4: CAS No. 5858-39-9). (The chemical structures for these compounds are shown in Figure 1.1)

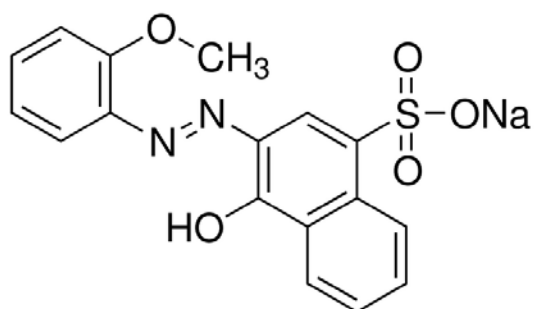
- to determine if pretreatment of dyes by iron reduction is necessary.
- to optimize SBP catalyzed removal of aniline (CAS No. 62-53-3) and *o*-anisidine (CAS No. 90-04-0) as models for pretreatment products. (The chemical structures are shown in Figure 1.2)
- To determine the impact of additives on treatment efficiency.
- To develop a new process for the treatment of azo-dyes through iron reduction followed by SBP catalyzed treatment of their recovered anilines in the presence of additives that have the potential to decrease enzyme demand.

1.6 Scope

- Investigated the potential for SBP to decolorize waters containing COG & AR4 directly. Otherwise, Fe reduction was implemented to reduce 1mM of the dyes to their aniline intermediates.
- Optimized the decolourization of waters containing these dyes with SBP with respect to pH, H₂O₂ molar ratio, SBP concentration and reaction time.
- Optimized the removal of aniline and *o*-anisidine with SBP with respect to pH, H₂O₂ molar ratio, SBP concentration and reaction time.
- Evaluated the effect of different additives [polyetheylene glycol (PEG), sodium dodecyl sulfate (SDS), Triton X-100, sodium dodecanoate (SDOD)] on optimum enzyme concentration.

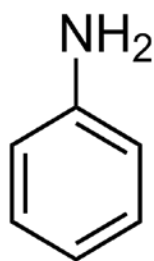


Crocein Orange G

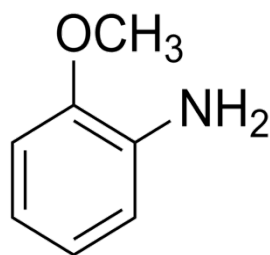


Acid Red 4

Figure 1.1 Azo-dyes selected for this study



Aniline



***o*-Anisidine**

Figure 1.2 Anilines selected for this study

CHAPTER 2

Literature Review

2.1 Anilines

2.1.1 Aniline

Aniline which is the simplest aromatic amine has been placed on Canada's priority substance list (PSL) since 1988, making it obligatory for companies to provide information on the release and disposal of the chemical via the National Pollutant Release Inventory (NPRI) list. Similarly, the U.S. EPA Toxics Release Inventory (TRI) also lists aniline as a toxic substance. Aniline is mainly used as an intermediate in various chemical productions such as rubber, herbicides, pesticides, dyes and pigments, and especially azo-dyes (U. S. EPA, 1994a). Six hundred and twenty seven kilotons of aniline was produced in the U.S. in 1992 (U.S. EPA, 1994a). In Canada, only one facility reported manufacturing 28 tonnes of aniline in 2007, as a by-product of chemical manufacturing (Government of Canada, 2010). Between 13 - 48 tonnes of aniline, as well as between 4 - 44 tonnes of N,N-diethylaniline and 3 to 8 tonnes of other aniline derivatives and their salts were imported in 2000 to 2007 (Government of Canada, 2010). In 2012, it was reported that over 786 tonnes of aniline was released or disposed of in the

U.S. and 61 tonnes of aniline was released or disposed of in Canada; however those facilities that produce, manufacture or use less than 10 tonnes of aniline per year do not have to report their releases (TRI, 2012; NPRI, 2012).

Aniline enters the environment through its industrial use. Sunlight can breakdown aniline in air, surface water and soil, while microorganisms can break it down in water and soil. It can easily penetrate groundwater because it does not bind well to soil (U.S. EPA, 1994b). It has been detected in a shallow aquifer known to be contaminated by coal-tar wastes, a Wyoming aquifer near an underground coal gasification site, a soil sample near Buffalo River in New York, and an air sample in Raleigh, NC (U.S. EPA, 1994a). In Ontario, Canada, groundwater samples collected near a landfill site and close to a chemical company had a concentration of aniline that ranged from 0.01 mg/L to 300 mg/L (Government of Canada, 1994).

Exposure to aniline through inhalation, ingestion or skin contact can cause toxic effects in humans. Extended exposure to aniline causes an increase in methaemoglobin production and a decrease in hemoglobin as well as adverse splenic effects and erythrocyte damage (U.S. EPA, 1994a; Government of Canada, 2010). Symptoms associated with anoxia resulting from methemoglobinemia include headache, light-headedness, ataxia, and weakness (Beard and Noe, 1981). An occupational study of workers exposed to 1.3 to 2.75 mg/m³ (0.19-0.39 mg/kg/day) aniline for 3 to 5 years showed a decrease in hemoglobin which was also present upon re-examination one year after (U.S. EPA, 1994a). In a 1972 study by Jenkins et al., volunteers given single aniline oral doses ranging from 5 to 65 mg developed increased methaemoglobin production at doses 25 mg or higher. It was also concluded by the author that humans were more sensitive than rats

to aniline exposure. A person who was exposed to aniline by sitting on a contaminated seat experienced dyspnea, fatigue and dizziness associated with a methemoglobin level of 53%. In one fatal case of aniline exposure, liver cirrhosis and atrophy were reported (U.S. EPA, 1994a). Slight symptoms occurred in several hours of exposure to 7-53 ppm aniline through inhalation and serious disturbances in inhalation exposure for 1 hour of 100-160 ppm (ACGIH, 1991). Aniline industry studies on aquatic life have shown that it is highly toxic to aquatic life (U.S. EPA, 1994b). The U.S. EPA classifies aniline as highly toxic with a probable oral lethal dose in humans at 50 to 500 mg/kg body mass. While the National Institute of Occupational Safety and Health set the immediately dangerous to life or health limit (NIOSH IDLH) at 381 mg/m³ inhaled (U.S. EPA, 2013a).

2.1.2 *ortho*-Anisidine

o-Anisidine is an aromatic amine whose chemical structure consists of an aniline with a methoxy functional group. It is found on U.S. EPA TRI list, as well as Europe's Directive 76/769/EEC restricting its use and marketing (European Commission, 2002). It is classified as a cat. 2 carcinogen, one which should be regarded as if it is carcinogenic to man, and a cat. 3 mutagen, substances which cause concern for man owing to possible mutagenic effects by EU RAR 2002 Risk of Assessment Report (European Commission, 2002). In 2002, *o*-anisidine was included in Directive 2002/61/EC which banned certain azo dyes, which can break down under reductive conditions to release certain aromatic amines, from being used in regular skin contact consumer goods (Puntener and Page, 2004). It has also been classified by IARC as a group 2B possible human carcinogen (IARC, 1999). *o*-Anisidine has been not produced in Canada and it was last produced in

the U.S. in 1957 (NTP, 2011). In 1995, it was produced in Armenia, China, France, Germany, India, Japan, Ukraine and the United Kingdom and as of 2009 it has only been produced by six manufacturers worldwide (IARC, 1999; NTP, 2011). In 2002, imports of *o*-anisidine into the U.S. were up to 227 tonnes (NTP, 2011). *o*-Anisidine is mainly used in dye and pigment production, as well as in pharmaceuticals, as a corrosion inhibitor for steel and as an antioxidant for polymercaptopan resins (IARC, 1999; NTP, 2011). According to TRI 110 kilograms were released in 2012 (TRI, 2012).

Presence of *o*-anisidine in the environment is a result of oil refinery and chemical plant wastewater as well as cigarette smoke. It is expected to degrade in air, with a half-life of 6 hours (NTP, 2011). In surface water, it binds to sediment and volatilizes with a half-life of 31 days in streams and 350 days in lakes, with little potential of aquatic bioaccumulation (NTP, 2011). In soil it binds to humic materials and is expected to biodegrade at low concentrations (NTP, 2011). Occupational exposure can occur through their manufacture or their use as a chemical intermediate (IARC, 1999).

Exposure to humans is mainly through inhalation, skin contact and ingestion (NTP, 2011). In studies on cats, which have similar capacity to form methaemoglobin as humans, it was able to induce methaemoglobin production with a single injection of 7.7 mg/kg (McLean et al., 1969; European Commission, 2011; Stocker, 2002). Other studies have shown that repeated oral ingestion resulted in haemolytic anemia, changes in enzyme parameters and organ weights (liver, kidney, and spleen) (Stocker, 2002). Workers exposed to 2 mg/m³ for 3.5 hours/day for 6 months developed headaches, vertigo and increased methaemoglobin (European Commission, 2011). In an oral exposure study of mice and rats to *o*-anisidine hydrochloride, tumors of the urinary

bladder occurred, and it was assumed that the carcinogenic affect was due to *o*-anisidine (Stocker, 2002) (European Commission, 2002). Mutagenicity of *o*-anisidine was shown to be positive in *in-vitro* tests and negative in *in-vivo*, leading the 2002 EU RAR to categorize it as a class 3 mutagen (European Commission, 2002). Thus, it was concluded that *o*-anisidine is a genotoxic carcinogen. The U.S. EPA sets the NIOH IDLH for *o*-anisidine inhaled at 50 mg/m³ (U.S. EPA, 2013b).

2.2 Azo-Dyes

The history of dyes goes back to prehistoric times, when natural vegetable extracts and animal products were used. In the mid-19th century, the synthetic dye industry started and it was not until 1876 that the first azo-dye was synthesized and marketed as London Yellow (Bafana et al., 2011; Morris and Travis, 1992). In 2008, the world market of dyes was valued at US \$16 billion and is projected to grow at a rate of 3.6% from 2013 to 2018 (Bafana et al., 2011; Market Watch, 2013). Azo-dyes are considered to be the largest group of colourants produced in the world. Their azo linkage (-N=N-) and aromatic components allowed for a diverse and brilliant combination of colours. They are easily synthesized, have a vast structural diversity, high molar extinction coefficient, and medium-to-high fastness properties with respect to light and wetness (Banafa et al., 2011). Their applications are used in many industries such as dyeing, medicine, ink, cosmetics, food and paints (Banafa et al. 2011).

2.2.1 Classes of Azo-Dyes

Azo-dyes can be further sub classified as **basic, disperse, solvent, acid, or direct** (Chudgar and Oakes, 2001; Spadaro, 1994). **Basic** dyes are cationic and are the simplest

synthetic dyes. They can be applied to synthetic fibers that contain large numbers of anionic sites such as modified acrylics, modified nylons, modified polyesters, leather, unbleached papers, and inks. **Disperse** dyes are hydrophobic and have a low water solubility. They are applied to hydrophobic fibers such as polyester and nylon. **Solvent** dyes are insoluble in water and only soluble in non-aqueous solution. They are further split into oil-soluble and spirit soluble and are used in petroleum products, lacquers, plastics, printing inks and other hydrophobic media. **Acid** dyes are anionic and are soluble in water due to their sulfonate substituents. Their three classes are those that are applied from a dyebath, those that are applied in the presence of metal (mordant), and those applied in the premixed metal-dye-complex (premetallized). **Direct** dyes are also anionic dyes that contain sulfonate groups. However, electrolytes are used in the aqueous dye bath to be applied on cotton and other cellulosic fibers. They have a larger size than acid dyes and usually contain a bi-azo structure (Chudgar and Oakes, 2001; Spadaro, 1994).

2.2.2 Azo-Dyes in the Environment

Azo-dye import into Canada and their use above reporting thresholds has led Environment Canada to view them as potential aquatic and terrestrial environment contaminants. They can be released into the environment from industrial facilities that dispose their wastewater into local wastewater treatment plants or aquatic environment directly (Environment Canada, 2012). Azo-dyes are persistent in the environment because of their high degree of chemical and photolytic stability. Their azo bonds and sulfonate groups are attributed for their recalcitrance, since they are not synthesized in the biosphere and are considered xenobiotic (Banafa et al., 2012; Kulla et al, 1983). This

also accounts for aerobic wastewater treatment plants' inability to treat azo-dyes, especially sulfonated-azo-dyes (Kulla et al., 1983). However, dyes applied to products do fade with time and can separate from the product upon washing. A 1980s study of Quebec's Yamasaka River identified the presence of three azo-dyes with the largest concentration downstream of a textile mill. Sediments, 6 km downstream, showed a mutagenic degradation product as a result of the azo-bond cleavage (Maguire and Tkacz, 1991). In 1984, two azo-acid-dyes, Acid Orange 156 CAS RN 57741-47-6 and Acid Red 266 CAS RN 57741-47-6, were found in the wastewater effluent at concentrations of 48 and 12 µg/L entering the Coosa River Basin in Alabama (Tincher, 1986).

Once released into the environment, the fate of azo-dyes depends on their own properties and those of the environment. Azo-dye degradation is usually done by oxygen-insensitive azoreductases. The aromatic amines resulting in aerobic conditions are mineralized; whereas azo-bonds are cleaved by various bacteria under anaerobic conditions. The aromatic amines are accumulated instead of mineralized in an oxygen deprived environment (Kulla et al., 1983). A study by Kulla (1983), showed that substituting (electron-withdrawing) sulfonate groups for carboxy groups in azo-dyes resulted in less degradation, making them less susceptible to oxidative catabolism. Thus, sulfonated azo-dyes could be decolourized by microorganisms under anaerobic conditions. In 2009, Hsueh et al. demonstrated that the position of the electron-withdrawing group in azo-dyes affected decolourization; with *para* showing greater decolourization than *ortho* which was greater than *meta*. It was also shown that azo-dyes with electron-withdrawing groups, eg. sulfonate group, are decolorized more readily than

those with electron-releasing groups, e.g. -NH-triazine group, and those with more electron-withdrawing groups were decolorized much faster (Hsueh et al., 2009).

2.2.3 Azo-Dye Health Effects

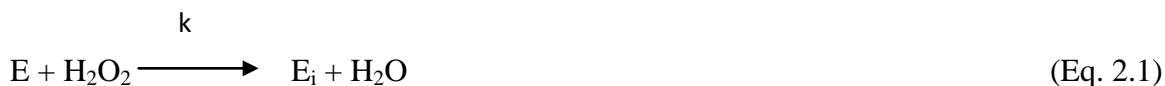
The government of Canada has identified 358 azo- and benzidine-based substances, including azo-dyes, as priorities for action and considered as priority pollutants under Canada's Chemical Management Plan based on ecological and human health concerns (Environment Canada, 2012). In 1994, Germany was the first European Union (EU) country to place in effect legislation on the use of certain azo-colourants under the German Consumer Good Ordinance, restricting their use in consumer products intended for more than temporary contact with skin. The EU enacted similar legislation in 2002 under Directive 2002/61/EC, which later on was replaced by Commission Regulation (EC) No 1907/2006 and amended to (EC) 552/2009 on June 22, 2009, in Annex XVII of the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) database (EU, 2006; EU, 2009). The legislation restricts the use of azo-dyes which by reductive cleavage of azo linkage may release one or more aromatic amines listed in Appendix 8 in detectable concentrations (i.e. 30 mg/kg) in the finished articles or in the dyed parts. The restriction applies to textile and leather articles that may come into direct and prolonged contact with human skin. In 1996, the Significant New Use Rule (SNUR) was added by the US EPA to control benzidine-based chemical substances manufacture, import and processing. An action plan was put in place by the US EPA in 2010, in which 48 dyes derived from benzidine and congeners (azo-dyes) would be investigated, of those 9 were proposed to be added to the SNUR list in 2012. Other countries such as India, Japan, Australia and New Zealand have also issued their own legislations with regards to

azo-dyes (Environment Canada, 2012). The major health concern of azo-dyes is the ability of azo-group cleavage and the production of genotoxic and carcinogenic aromatic amines as a result. Some azo-dyes have also been shown to have haematological effects, with mutagenic potential (Environment Canada, 2013). These factors show the importance for treatment of wastewater effluent containing azo-dyes and the potential aromatic amines from their azo-reductive cleavage.

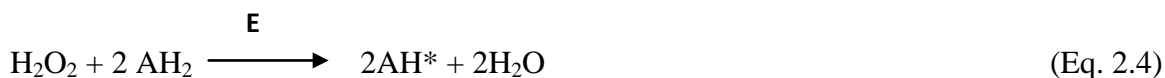
2.3 Peroxidases

Peroxidases belong to the class of oxidoreductases enzymes. They are widely distributed in nature, abundant in microbial and plant sources but not so much in mammalian cells. They catalyze the oxidation of a wide range of substrates in the presence of hydrogen peroxide (Dunford and Stillman, 1976). Peroxidases are used in many industries and processes ranging from indicators in the food industry, protein engineering, recombinant protein expression and as catalysts for the removal of aromatics from wastewater (Flock et al., 1999; Ryan et al., 2006; Regalado et al., 2004). Their wide use is due to their high redox potential, and structural properties giving them relatively high thermal stability and wide distribution (Demarche et al., 2012; Regalado et al., 2004). Aromatic compounds, which are a major class of pollutants, can be removed by polymerization using peroxidase enzymes. Hydrogen peroxide acts as the electron acceptor catalyzing peroxidase oxidative polymerization of phenols, anilines, azo-dyes and other aromatics to insoluble oligomers (Dunford and Stillman, 1976). These oligomers serve as further hydrogen donors leading to further polymerization, and ultimately transforming water-soluble aromatics to water-insoluble compounds. These compounds can be easily

removed through sedimentation and filtration (Kilbanov and Morris, 1981). This polymerization process can be shown by the following equations (Dunford, 2010; Nicell et al., 1993).



The overall enzymatic reaction



These equations can be expressed in a cycle starting with native enzyme (E) which upon a 2-electron oxidation by hydrogen peroxide forms Compound I (E_i). In turn Compound I accepts an aromatic compound (AH_2) in its active site which undergoes one-electron oxidation releasing a free radical (AH^*). The Compound I enzyme is further reduced to Compound II (E_{ii}) which oxidizes a second aromatic compound releasing a second free radical, and returning to its native state (E). The free radicals combine to form dimers and then undergo further enzymatic conversion to radicals that form trimers, tetramers, oligomers and even polymers (Nicell et al., 1993; Dunford, 2010; Ibrahim et al., 2001).

Inactivation and inhibition of active enzymes results from side reactions that occur in this system (Nicell et al., 1993). Evidence of a reversible intermediate created during the formation of Compound I was first seen by Baek and Van Wart (1989). This resulted in the formation of an $E-H_2O_2$ complex and an oxidized enzyme which was referred to as Compound 0. Suicide inactivation as denoted by Arano et al. (1990) is formed in the

absence of substrate and in excess hydrogen peroxide, and occurs in one of two possible ways. In the first possibility, Compound II is oxidised by the excess hydrogen peroxide to Compound III (E_{iii}). Although Compound III is catalytically inactive, it does decompose back to the native enzyme. However, the decomposition is so slow that it can be assumed that once Compound III is formed it renders that enzyme inactive.



The second way proposed by Arano et al. (1990), involves an irreversible inactivated intermediate Compound P_{670} . At hydrogen peroxide concentrations above 1 mM P_{670} is dominant.

Klibanov et al. (1983) proposed that inactivation resulted from the return of a free radical to the active site, blocking that site and preventing further catalysis. Nakamoto and Machida (1992), alternatively suggested that inactivation was a result of end-product polymer adsorption of enzymes, blocking substrate access to the active site.

The most investigated peroxidase is the horseradish peroxidase (HRP) (Dunford, 2010). Klibanov et al. (1980, 1981) were the first to use HRP to remove over 30 phenols and aromatic amines from water, achieving 99% removal for some pollutants. However, it has been shown that HRP has a short catalytic lifetime due to enzyme inhibition and reaction temperature. It is active mainly between 5-55°C and are rapidly inactivated at temperatures above 65°C, and also is inhibited at low pH (Caza et al., 1999; Flock et al., 1999, Yu et al., 1994). The main disadvantage in the use of HRP is the high cost for extracting and purifying, limiting its availability in large quantities at a price that would be feasible for wastewater treatment (Bassi et al., 2004; Al-Ansari et al., 2009). These

disadvantages paved the way for looking at other peroxidases such as soybean peroxidase (SBP).

2.3.1 Soybean Peroxidase

Soybean peroxidase (SBP) is a hemoprotein oxidoreductase enzyme that is extracted from the seed coats hull of soybeans (Gizen et al., 1993). It is considered to have a broad pH and temperature stability. SBP is active at pH as low as 2.0 and temperatures of 70°C, even at temperatures of 85°C the half life is 2.5 hours (McEldoon and Dordick, 1996; Ryan et al. 2006). The soybean crop is estimated to grow on 6% of the world's arable land with the highest percentage of increase in comparison with other crops. In 2008, soybean production reached 230 million tonnes, more than 12.5 times production in 1960 (Hartman et al., 2011). Since the seed coats of soybeans are considered as waste products, their hulls provide an inexpensive and abundant source of enzyme that can be commercially used in wastewater treatment (Nicell, 2003; Hailu et al., 2010). Previous studies have proven SBP to be very effective in treating aromatic compounds, as shown in Table 2.1.

Table 2.1: Soybean peroxidase treatment of various aromatic compounds

Enzyme Source	Substrate	Reference
SBP	Phenols, chlorophenols, & cresols, bisphenol A	Caza et al., 1999
	Cresols	Biswas, 1999
	Phenol & 2-chlorophenol	Flock et al., 1999
	Phenol	Wright & Nicell, 1999
	Phenol	Kinsley and Nicell, 2000
	Aniline & toluidines	Mantha, 2001
	Phenol	Wilberg et al., 2002
	2,4-dichlorophenol	Kennedy et al., 2002
	Phenol & chlorophenols	Bassi et al., 2004
	Dyes: Direct Yellow 11 & Basazol 46L	Knutson et al., 2005
	Phenol	Gomez et al., 2006
	Phenol	Trivedi et al., 2006
	Diaminotoluenes	Patapas et al., 2007
	Phenylendiamines & benzendiols	Al-Ansari et al., 2009
	Phenols in coal-tar wastewaters	Al-Ansari et al., 2010a
	2-mercaptobenzothiazole	Al-Ansari et al., 2010b
	Dye: Turquoise Blue G 133	Marchis et al., 2011

2.4 Peroxidases in Wastewater Treatment

Klibanov et al. (1980) were able to show that the addition of aromatic compounds that were easily removed by enzymatic treatment of a wastewater, improved the enzymatic precipitation of those with lower removal efficiencies. Moreover, Klibanov (1982) stated that substances that were not substrates of the enzyme were also precipitated in the presence of easily removed substrates. If true, this would make enzymatic treatment more feasible in real wastewaters, which are usually composed of many different pollutants. Since then, many other studies have looked at HRP for the treatment of aromatic compounds such as bentazon (Kim et al., 1998) 2,4-dichlorophenol (Laurenti et al., 2003), 4-chlorophenol (Nicell, 1994), phenols (Wagner and Nicell, 2002; Bodalo et al., 2006), aniline (Shan et al., 2003), azo dyes (Stibrova et al., 1996), dyes (Bhunja et al., 2001), acid azo-dye (Acid Black 10x) (Mohan et al., 2005), and textile dyes (Ulson de Souza et al., 2007).

Arthromyces ramosus peroxidase (ARP) has been shown to be a promising enzyme for wastewater treatment because of its low cost and potential for commercial availability (Biswas, 2004). Ibrahim et al. (2001) studied the treatment of phenol from real refinery wastewater sample and compared it with synthetic wastewater. The study showed that 95-99% of the same amount of phenol was removed by the same amount of ARP in both real and synthetic wastewater, meanwhile step-addition of H_2O_2 did not reduce the amount of enzyme required. ARP was shown to be effective at reducing azo-dyes, including COG and AR4, directly by Wang (2002). Azo-dyes, pretreated by zero-valent iron reduction, were converted to aromatic amines that were then treated successfully by ARP (Biswas et al., 2007). SBP treatment is seen as a better peroxidase choice than HRP

and ARP for treatment of wastewater because it is less susceptible to inactivation by H_2O_2 , and has greater potential for production in larger quantities at low costs (Al-Ansari et al., 2011).

Caza et al. (1999) demonstrated that purified SBP was able to remove 95% of various phenolic compounds from synthetic wastewater. Crude SBP, extracted from soybean hulls, was compared by Biswas (1999) with commercially purified SBP in the removal of cresols from synthetic wastewater. The study showed that crude SBP was more thermostable (up to 80°C), and had a higher H_2O_2 demand which increased with increased SBP addition than purified SBP. The comparison of the studies by Biswas (1999) and Caza et al. (1999) demonstrates that crude SBP was more efficient in removing cresols than purified SBP. Similarly, Flock et al. (1999) compared purified SBP to crude SBP in the treatment of phenolic compounds and concluded that crude SBP was more effective than pure SBP. Treatment of dyes with SBP was studied by Knutson et al. (2005). In this study, an azo-dye, Direct Yellow 11, and methine-dye, Basazol 46 L, were successfully treated by both HRP and SBP. However, SBP was considered more effective in oxidative dye removal.

Enzymatic treatment of aniline can produce polyaniline, a conducting polymer of high environmental stability and interesting electronic properties (Flores-Loyola et al. 2007; Shan et al., 2003; Regalado et al., 2004). They have a wide range of applications such as anticorrosive protection, optical display, photoelectrochemical cells and light emitting diodes (Raitman et al., 2002). Due to the fact that polyanilines have to be synthesized under acidic conditions, SBP treatment of aniline can yield a by-product that can be

diverted from disposal, thus decreasing cost of disposal and even creating a value added benefit (Regalado et al., 2004; Taylor et al., 1998).

Studies on SBP removal of aromatic compounds have to be optimised for several parameters including, temperature, pH, H_2O_2 concentration, enzyme concentration and reaction time. One of SBP's main advantages is the wide range of temperature (active at 70°C) and pH (2-10) under which the enzyme is active (Ryan et al. 2006). Equation 2.4, shows that stoichiometrically 0.5 moles of H_2O_2 are needed for 1 mole of substrate, i.e. a 1 to 2 ratio. Studies have shown that the actual stoichiometry is closer to 1:1 (Taylor et al., 1998; Ibrahim et al., 2001). It is assumed that this extra H_2O_2 consumption is due to the side reactions that form polymers (Nicell, 1991). However, one should be mindful that an excess of H_2O_2 can lead to lower removal efficiency likely due to inactivation of the enzyme (Nicell, 1991).

Certain compounds that are not substrates of peroxidase can be pre-treated and then treated enzymatically. Mantha et al. (2002) was able to treat nitroaromatics enzymatically by first employing zero-valent iron reduction to convert them to anilines and then treating anilines by crude SBP in a continuous-flow system. This method was also employed in the treatment of azo-dyes (Biswas et al., 2007; Wang, 2002).

According to Nicell (2003), the following six criteria that have to be met for an enzyme to be considered feasible for waste-treatment application:

1. Confirm the ability of an enzyme to selectively act upon the target substrate.
2. Enzyme be able to actively catalyse substrate under typical conditions.
3. Enzyme should be stable under required reaction conditions.

4. Enzymatic reactor systems should be simple to be accepted by potential industries.
5. Reaction products should be less toxic and more biodegradable or easier to treat in downstream applications than the original pollutant.
6. Enzymes must be commercially available.

Soybean peroxidase, due to its potential for affordable commercial availability, wide range of thermal and pH stability and ability to treat a wide range of aromatic substrates makes an excellent choice to be used for wastewater treatment.

2.4.1 Additives in Enzymatic Wastewater Treatment

Deactivation of enzymes is one of the major disadvantages of enzymatic treatment in wastewater, which results in large amounts of enzymes used and an increase in cost. The use of additives to aid in enzymatic treatment of wastewater was first suggested by Nakamoto and Machida (1992). They hypothesized that inactivation was due to end-product polymer hindering access to the enzyme active site. Their suggestion was to add proteins or hydrophilic synthetic polymers to suppress the enzyme inactivation. In their study, Nakamoto and Machida (1992) added polyethylene glycol (PEG) and gelatin during the HRP treatment of wastewaters containing 10 to 30 g/L phenol and were able to reduce the amount of enzyme down to 1/200 of the amount needed without additive. The amount of additive used was shown to increase proportionally with the increase in the amount of substrate in solution (Nakamoto and Machida, 1992). Wu et al. (1993) used the same approach as used by Nakamoto and Machida (1992) but applied it to more realistic wastewater concentrations of phenol, 1-10 mM (0.1-1 g/L) of phenol, and

optimized the reaction for pH, H₂O₂, HRP and PEG. The study showed that PEG significantly decreased the amount of HRP enzyme used at an optimal pH of 8.0 and H₂O₂ to substrate ratio of 1 to 1; however, PEG was more effective at higher phenol concentrations. The effect of PEG on HRP removal of phenolic compounds was also investigated in other studies (Nicell et al., 1995; Wu et al., 1997).

Addition of PEG (MM 3350) to ARP-treatment of phenol was also shown to be effective in enhancing enzyme activity (Ibrahim et al., 2001). Similar improvements were observed with its addition to laccase-treated bisphenol-A (Modaressi et al., 2005). However, the addition of PEG (MM 3350) to SBP for removal of phenol was marginally effective (Caza et al., 1999). Kinsley and Nicell (2000) showed that for improvement of enzymatic activity of SBP in the removal of phenol, PEG with a molecular mass of 35,000 has to be added.

Tonegawa et al. (2003) compared PEGs with different molecular masses as well as surfactants, rhamnolipid, Triton x-100, Tween 20, SDS and NP-40, in the treatment of phenols by HRP. All additives provided improved 2,4-dichlorophenol removal with enzyme by 60%, while the effectiveness of PEG was dependent on its molecular mass, with higher molecular mass (above 400) being more effective (Tonegawa et al., 2003). Flock et al. (1999) studied the effect of different detergents (SDS, Tween 20 and Triton X-100) on the removal of phenol and 2-chlorophenol with SBP. The study showed that, even at low concentrations (0.1 % w/v), the detergents significantly increased enzyme activity, over 3 fold for SDS and Tween 20 and 2 fold for Triton X-100. Also, SBP was shown to remain active even under high concentrations of detergents (up to 20% w/v). Similarly, the removal of phenol by *Coprinus cinereus* peroxidase was enhanced by the

addition of Triton X-100, Triton X-405 and Tween 20, as much as high molecular mass PEG (3000). The addition of Span 20, SDS and lauryl trimethylammonium bromide (DTAB) also enhanced enzymatic removal of phenol but was dependent on pH (Sakurai et al., 2003). Al-Ansari et al. (2010a) were able to decrease the amount of SBP needed to achieve 95% removal of phenols in coal-tar wastewater by five-fold after the addition of SDS. In synthetic wastewater, the addition of Triton X-100 was able to reduce the amount of SBP needed for the removal of 95% of 1 mM phenol better than SDS (Al-Ansari, 2010a).

A recent study by Feng et al. (2013), in which crude SBP was used in the treatment of phenol, showed that SBP trapped in polymerized phenol precipitate remained active. This allowed for the recycling of precipitate which reduced the amount of fresh enzyme needed in the treatment. Triton X-100 was used in the study to reverse the immobilization of SBP on the phenolic precipitate, resulting in 5.9-fold higher activity. This implies that SBP inhibition can be reversed in the presence of Triton; however, it was observed that a small fraction of SBP remained on the precipitate. Steevensz et al. (2014) extended this work to a broad range of phenol concentration (1-10 mM) in synthetic wastewater as well as in a real wastewater sample. The study showed that the amount of Triton X-100 needed in the reaction increased linearly with that of the substrate concentration. Moreover, the addition of Triton X-100 decreased the amount of SBP needed by 10 to 13-fold.

The method by which additives improve enzymatic removal of aromatic compounds is not fully understood. Nakamoto and Machida (1992) theorized that enzyme inactivation was due to end-product polymers adsorbing the enzyme, which prevented its access to the

active site. Thus, PEG acted as a sacrificial polymer reacting with these polymeric products preventing them from hindering the enzyme active site and possibly precipitating it. Supporting their theory they were able to show that PEG helped retain HRP in aqueous phase, whereas, without PEG addition HRP was precipitated with the polymeric product. In a recent study by Mao et al. (2013), evidence showed that HRP enzyme was inactivated by heme destruction. HRP-mediated phenol reactions in the study showed that iron releases from enzyme inactivation is reduced in the presence of PEG indicating that heme destruction is suppressed by PEG.

2.5 Zero-Valent Iron Reduction

Reduction of halogenated hydrocarbons by galvanized steel was investigated by Reynolds et al. (1990); however, Gillham and O'Hannsein (1994), motivated by the 1972 patent literature of Sweeny and Fisher, were the first to use iron for the degradation of chlorinated aliphatic compounds in aqueous solution. The use of zero-valent iron versus other metals is considered to be advantageous due to its availability at low cost and effectiveness in the degradation of a wide range of halogenated organic compounds (Gillham and O-Hannesin, 1994; Matheson and Tratnyek, 1994). The reduction of other chemical compounds by zero-valent iron followed: chlorinated pesticides (Sayles et al., 1997), nitro-aromatic compounds (Agrawal and Tratnyek, 1996; Mantha, 2001), nitrates (Cheng et al., 1997), metals (Cantrell et al., 1995), dinitrotoluenes (Patapas et al., 2007) and azo-dyes (Nam and Tratnyek, 2000; Cao et al., 1999; Wang, 2002; Biswas, 2004). Zero-valent iron treatment of wastewater is done under anaerobic conditions to prevent corrosion of iron. Under this condition, iron is a strong reducing agent, water acts as an

oxidizing agent by corroding Fe^0 to produce Fe^{2+} , OH^- , and H_2 gas (Reardon, 1995; Agrawal and Tratnyek, 1996) as shown in the following equation:



Azo-dyes treated by zero-valent iron are decolourized through the reduction of their azo linkages (Cao et al., 1999, Nam and Tratnyek, 2000). Weber (1996) concluded that the reduction is surface mediated, making it necessary for the substrate to have direct contact with iron surface. There are several steps involved in this electrochemical corrosive process. The first involves the adsorption of the substrate on the iron surface, followed by the oxidation of iron and reduction of the substrate and finally the desorption of the by-products from the iron surface (Biswas, 2004). Cao et al. (1999) suggested that the degradation of azo-dye by zero-valent iron is a two step reaction, the first being reversible. They observed that after some time some of the colour returned, indicating that the degradation solution was composed of the azo-dye, breakdown products and a transitional compound which would transform back to azo-dye.

Several factors have to be controlled to promote better reduction efficiency. These include iron surface area, pH, and mixing rate. Use of ultra-fine, nano-scale particles provides a larger iron surface area and thus more reactive sites (Choe et al., 2001). Cleaning the iron surface with hydrochloric acid (HCl) prior to the reaction enlarges the effective surface area by removing the inhibiting oxide layer of iron and thus promoting degradation. The rate of decolourization increases with the rate of mixing (Nam and Tratnyek, 2000). The use of carbonate buffer in the reaction slowed down the reduction

by inhibiting access to the iron surface (Lavine et al., 2001). A lower pH increases degradation yield as well as decreases the reaction time (Cao et al., 1999). However, it must be noted that at pH concentrations below 5.0, aniline was observed to adsorb onto iron, which is unfavourable since it would require further treatment for aniline desorption (Agrawal and Tratenyak, 1996; Mantha, 2001).

The decolourization of azo-dyes by zero-valent iron has been shown to be feasible in wastewater treatment. However, the reduction products of azo-dyes through this process are aromatic amines which are more toxic and thus zero-valent iron reduction has to be used as a pre-treatment method in conjunction with amine treatment methods. In this research, soybean peroxidase enzymatic treatment was used for the treatment of parent anilines, as well as anilines produced as breakdown products of zero-valent iron pre-treatment of azo-dyes, along with direct enzymatic treatment of azo dyes to determine the optimal methods of treatment.

CHAPTER 3

Materials & Methods

This chapter presents the experimental procedures and analytical techniques used in this study.

3.1 Materials

3.1.1 Anilines and Azo Dyes

Aniline and *o*-anisidine (99% purity) were purchased from Sigma-Aldrich Chemical Company (Oakville, ON). Azo dyes Acid Red 4 (AR4, 45% purity) and Crocein Orange G (COG, 90% purity) were purchased from MP Biomedicals (Solon, OH).

3.1.2 Enzymes

Crude dry solid SBP (E.C. 1.11.7, Industrial Grade lot #18541NX, RZ = 0.75 ± 0.10 , activity ≈ 5 U/mg) was obtained from Organic Technologies (Coshocton, OH). Dry solid bovine liver catalase (E.C. 1.11.1.6, lot #120H7060, activity $\approx 19,900$ U/mg) was purchased from Sigma Chemical Company Inc. (Oakville, ON). The enzymes were stored at -15°C , while the sub-stock solutions prepared were stored in 4°C .

3.1.3 Additives

Sodium dodecyl sulfate (SDS), sodium dodecylbenzenesulfonate (SDBS) and polyethethylene glycol (PEG, average molar mass of 3350 g/mole) were purchased from Sigma Chemical Company Inc. (Oakville, ON). Triton X-100 was purchased from Alphachem (Mississauga, ON). SDOD (sodium dodecanoate) (99-100% purity) was purchased from Sigma-Aldrich Inc. (Oakville, ON).

3.1.4 Reagents

2,4,6-Trinitrobenzenesulfonic acid solution (TNBS) (1.0 M in H₂O) was purchased from Sigma Chemical Company Inc. (Oakville, ON) and was stored at -15°C. 4-AAP (4-amino-antipyrine) was obtained from BDH Inc. (Toronto, ON) and stored at room temperature. Hydrogen peroxide (30% w/v) was purchased from ACP Chemicals Inc. (Montreal, PQ) and stored at 4°C.

3.1.5 Buffer and Solvents

Analytical grade monobasic and dibasic sodium phosphate, sodium acetate, sodium bicarbonate, sodium carbonate, concentrated hydrochloric acid, glacial acetic acid and 95% ethanol (anhydrous) were purchased from ACP Chemicals Inc. (Montreal, PQ). HPLC grade methanol was obtained from Fisher Scientific Co. (Ottawa, ON).

3.1.6 Others

Syringe filters (0.2 µm, non-sterile) were purchased from Gelman Laboratories (Mississauga, ON). BD Luer-Lok Tip 10 mL and 3 mL syringe were obtained from Dickinson and Company (Franklin Lakes, NJ). Iron Filings (approx 40 mesh), and

Cobalt Chloride were obtained from Fisher Scientific Company (Ottawa, ON). Fisherbrand Spinbar Teflon coated magnetic stir bars (various sizes) and Fisherbrand P8 Qualitative filter paper were purchased from Fisher Scientific (Ottawa, ON). Pipetman adjustable volume pipettes (200 μ L, 1000 μ L, 5 mL) were purchased from Mandel Scientific (Guelph, ON).

3.2 Analytical Equipment

3.2.1 UV-VIS Spectrophotometry

Two spectrophotometers were used to quantify azo dyes and anilines through direct absorbance or colorimetric methods. One was a Hewlett-Packard (model 8452A) Diode Array Spectrophotometer (λ range of 190 -820 nm and 2 nm resolution) controlled by a Hewlett Packard I/O card interfaced with a PC. The other was an Agilent 8453 UV-Visible spectrophotometer (λ range of 190 -1100 nm and 1 nm resolution) controlled by a Hewlett Packard Vectra ES/12 computer. Quartz spectrometer cuvettes with 1 cm path length were purchased from Hellma (Concord, ON).

3.2.2 HPLC (High Performance Liquid Chromatography)

Aniline samples were analyzed and compared to the colorimetric technique by HPLC from Waters Co. (Mississauga, ON) with a Model 2487 dual wavelength absorbance detector, Model 1525 binary HPLC pump and Model 717 autosampler operated by Breeze 3.3 software.. A Waters Symmetry C₁₈ reverse phase column (5 μ m, 4.6 X 150 mm) column was used.

3.2.3 Total Organic Carbon(TOC) Analysis

Carbon content of solutions was determined by a Shimadzu TOC-V CSH Total Carbon Analyzer, purchased from Shimadzu Scientific Instruments (Columbia, MD). The total organic carbon was calculated by the difference of total carbon (TC) and inorganic carbon (IC). Both TC and IC were detected by a non-dispersive infrared spectrophotometer. All samples were micro-filtered before injection. Standard curves for both TC and IC were selected from the machine database.

3.2.4 Sonicator

Mixing of azo-dyes pretreated by zero-valent iron was done by a Sonicator SC-101TH sonicator (50 /60 Hz, 2.3 Amps) from Sonicator Instrument Corporation (Copiague, NY).

3.2.5 pH Measurement

An EA940 pH meter with stainless steel micro pH probe was purchased from London Scientific (London, ON). Calibration buffers of pH 4.00, 7.00, and 10.00 were purchased from BDH Inc. (Toronto, ON).

3.2.6 Other Equipment

Model K-550-G vortex mixer (50/60Hz, 0.5 Amps) was purchased from Scientific Industries, Inc (Bohemia, NY). VWR magnetic stirrers VS-C10 (50-60 Hz, 30 Watts) were purchased from VWR International Inc. (Mississauga, ON).

3.3 Analytical Techniques

3.3.1 Colour Reduction

Stock solutions of AR4 and COG were made up to 2 mM with deionized water. Subsequently, a 1 mM stock was made from that stock solution. A 20-fold dilution of the 1 mM stock was analyzed by UV-VIS spectrophotometer to determine optimum wavelength (λ_{max}) for maximum absorbance for both dyes. In order to determine the amount of colour remaining after zero-valent iron pre-treatment or enzymatic treatment, both initial colour (A_i) as well as final colour (A_f) were measured at λ_{max} .

$$\text{Percent color remaining} = 100 * (A_f / A_i)$$

3.3.2 Anilines Colorimetric Assay

A TNBS test as per Al-Ansari (2008) was used to measure both parent anilines and anilines produced by Fe reduction of azo-dyes AR4 and COG. The reaction of anilines with TNBS in the presence of phosphate buffer of pH 7.4 and sodium sulfite generated a yellow chromophore. The colour intensity was proportional to the concentration of the anilines. Both the λ_{max} and time for colour development were dependent on the substrate and thus experiments were conducted to determine these parameters. Samples were made in a 1 mL cuvette and included 100 μL of 10 mM TNBS, 100 μL of 0.2 M phosphate buffer, 100 μL of 20 mM sodium sulfite, and 700 μL between sample and water volume; whereas for the blank 700 μL of water was used. The UV-VIS spectrophotometer was blanked with the sample blank and then set up to measure a range of wavelength (300-700 nm) every 2.5 minutes until maximum absorbance was reached.

Standard curves were plotted for substrate concentrations of 0.005 mM to 0.04 mM to be used in determining concentrations of anilines in the experiments.

3.3.3 Aniline Analysis by HPLC

HPLC was used to determine the accuracy of TNBS method in quantifying aniline concentrations. As per Steevensz (2008) aniline standard was run under isocratic conditions with 50% methanol (pump A) and 50% 20 mM phosphate buffer at pH 7.4. Flow rate was set to 1.0 mL/min 10 μ L volumes were through unheated column. UV detector was set to $\lambda_{\text{max}} = 280$ nm and peak retention time was observed at approximately 3.3 minutes. Standard curve was created using aniline concentration range of 0.1 - 1 mM and was used in determining unknown concentrations.

3.3.4 Enzyme Activity Assay

The catalytic activity of SBP was measured through a colorimetric assay as per Wu et al. (1997). In this study, 1 unit of activity is defined as the number of micromoles of H_2O_2 converted per minute at pH 7.4 and at room temperature. Thus, the rate of the catalyzing reaction was compared with the rate of uncatalyzed reaction. The assay included a reagent which used phenol, 4-aminoantipyrine (4-AAP) and H_2O_2 in excess to ensure that the initial rate of reaction was directly proportional to the concentration of enzyme activity. After the addition of 950 μ L of the reagent to 50 μ L of SBP in the cuvette to provide proper mixing, a reaction produced a pink chromophore with a maximum absorption at $\lambda_{\text{max}} = 510$ nm and an extinction coefficient of $6000 \text{ M}^{-1} \text{ cm}^{-1}$. The initial rate of colour formation in the first 30 seconds was monitored by the Agilent

spectrophotometer and activity results were calculated by using the kinetic rate calculation function built into the software.

3.3.5 TOC Analysis

TOC analysis was done on batch reactor experiments involving parent aniline compounds. 20 mL samples were collected and micro-filtered. The machine was allowed to start up and run with 3 milli-Q water injections to make sure the system was properly purged. Each sample was then measured for both TC and IC with the difference being TOC. 3 injections were used for each reading and the average was recorded.

3.4 Experimental Procedures

The experimental procedures used in this study are presented in the following three subsections. All experiments were conducted in batch reactors which were run in triplicate at room temperature (20 - 22 °C). The average values of these three readings are presented, with error bars representing the standard deviation.

3.4.1 Enzymatic Treatment of Anilines and Azo-Dyes

Batch reactors were set up to optimize SBP enzymatic treatment of both aniline and *o*-anisidine for 95% removal as well as optimal removal of azo-dyes AR4 and COG. Parameters investigated were: pH, H₂O₂ concentration, enzyme concentration, and reaction time. All experiments were conducted in 20 mL glass vials with each batch receiving 1 mM substrate. Acetate or phosphate buffers were used to cover pH of 3.5 - 7.5 for anilines, and 3.5-9.4 for azo-dyes. H₂O₂ concentration was varied from 0.5 - 3 mM for anilines and 1 - 5 mM for azo-dyes. SBP concentrations used depended on the

substrate a starting point of 0.17 U/mL was used for anilines and 3 U/mL SBP for azo-dyes and changed by increasing or decreasing concentration to achieve 95% removal. A teflon-coated magnetic stir bar was placed in the mixture and the vials were then placed on a magnetic stirrer to allow for thorough and continuous mixing. All reactions were run for 3 hours and were stopped by adding 100 μ L of catalase stock solution, which broke down H_2O_2 to water and oxygen. The samples were then microfiltered and analyzed by TNBS test explained in Section 3.3.2. Once various parameters were optimized for 95% removal conditions, experiments were conducted on enzymatic treatment of anilines to determine reaction time effect and kinetics by monitoring anilines removal over 3-hour period.

3.4.2 Additive Effect

Batch reactors were run to determine the effect of additives on enzymatic treatment. Various additives in a range of 10 - 200 mg/L were added to 20 mL glass vials as discussed in Section 3.4.1 and optimized and analyzed accordingly.

3.4.3 TOC

Batch reactors (25 mL) were run in tap water with 1 mM substrate, optimal H_2O_2 :substrate ratio, optimal SBP dose and optimal additive dose gathered from previous experiments. No buffer was added and the pH was not adjusted, the pH of the water was approximately 7.0. The reaction was stopped after 3 hours, the batch samples were then microfiltered and analyzed for aniline remaining as described in Section 3.3.2 and TOC remaining as described in Section 3.3.5. Standard curves were prepared for TOC of the

additives, SBP and aniline in order to assist in calculating theoretical values versus the real sample values measured by the TOC analyzer.

3.4.4 Zero-Valent Iron Pretreatment of AR4 and COG

Iron preparation: Iron filings (40 mesh) used in this experiment were first pretreated with acid to remove metal oxides from the surface allowing for more surface area contact with the substrate (Agrawal and Tratnyek, 1996). Iron filings were placed in a glass vial and soaked for 20 minutes in 10% HCl. To remove the metal oxides and all chlorides, the iron was then washed four times with 15 mM carbonate buffer (pH 9.5) made anaerobic by including 1 mM sodium sulfite. The iron was then washed four times with a 20 mM sodium sulfite solution (with 0.1% w/w cobalt chloride with respect to sodium sulfite) to remove excess alkalinity and prevent contact with oxygen. Finally, the iron was stored in this solution to maintain anaerobic conditions.

Colour Reduction: Batch reactor experiments were set up to decolorize AR4 and COG by cleaving the azo-linkage by zero-valent iron reduction, breaking them to their simpler compounds. Experiments were set up to optimize for amount of Fe needed, and time to achieve optimal colour reduction. Experiments were carried out in 40 mL screw cap glass vials. The appropriate amount of iron (0.5 - 2 g) was placed in 1mM azo dye solution made anaerobic by using sodium sulfite (with 1% w/w cobalt chloride). Tap water left out overnight to dissipate any chlorine was used in these batch runs, since tap water has the advantage of having buffering capacity close to neutral. The vials were placed in a sonicator water bath and the sonication was allowed for the required period of time (monitored for 2 hrs, every 15 minutes) to ensure proper mixing and contact with

Fe. After the reaction the vials were placed on a magnet to allow for iron particle settling and then were microfiltered. Samples were then analyzed for both reduction of colour as well as anilines produced by UV-VIS spectrophotometer analytical techniques in Sections 3.3.1 and 3.3.2.

Enzymatic treatment of anilines produced in azo-dye by Fe^0 : These experiments were conducted to determine the difference between enzymatically treated parent anilines and that of Fe^0 reduction products.

In 20 mL batch reactors 0.5 mM Fe^0 reaction anilines product was added and subjected to enzymatic treatment using varying SBP concentrations. The pH ranged from 4 to 8 with varying H_2O_2 concentrations (0.5 - 2 mM) to achieve 95% substrate removal. The enhancing effect of additives was also investigated as per Section 3.4.2, and all analysis was conducted via UV-VIS spectrophotometer as per Section 3.3.2.

3.5 Sources of Error

Experimental accuracy and reliability are affected by two types of errors systematic and random. Errors that include human error or equipment are considered random. To minimize these errors, experiments were conducted in triplicates, and the average taken and a standard deviation was plotted on the graphs. Those graphs that do not show error bars have a standard deviation below 1%, which would be hidden by the icons. Furthermore, the same pipetes, glass ware and other equipment were used in all experiments to minimize cross-contamination. Systematic errors are the product of improper experimental design, and analytical techniques' or instruments' inaccuracy. To minimize these errors, equipment were calibrated, and analytical instruments were tested

for known concentrations regularly. Experimental protocol and procedure was carefully followed and compounds that can easily degrade were prepared fresh for each experiment. The factors that may have contributed to the some errors in my study were associated with AR4 which had a purity of 45%; the dye with 90% purity, COG, had a significantly lower percent error.

CHAPTER 4

Results and Discussion

The aim of this chapter is to discuss how enzyme catalyzed removal of anilines and azo-dyes were optimized. Several parameters were investigated including, pH, H₂O₂ concentration, and SBP concentration. The experiments were designed to test the effect of various parameters in the presence of limited SBP concentration so that stringent conditions existed to clearly determine effect. Additives were used to minimize the amount of enzyme needed to achieve 95% removal of substrates by polymerization followed by filtration. Pre-treatment of azo-dyes with zero-valent iron was investigated as a way to achieve improved decolourization efficiency as well as possibly reducing the amount of enzyme needed. For the sake of comparison, an optimal removal efficiency benchmark was set as 95% for these compounds. Thus, in this study the mention of optimal refers to this benchmark, unless otherwise stated.

4.1 SBP Catalysis of Anilines

Zero-valent iron reduction of azo-dyes yields aromatic amines which exhibit toxic and carcinogenic effects (Stibrova et al., 1996; Biswas et al., 2007; Rodriguez Couto & Toca – Herrera, 2006). In this study, two anilines were studied, aniline and *o*-anisidine both of

which are by-products from the azo-linkage reduction of azo-dyes COG and AR4, respectively. Thus, it was important to optimize the enzymatic removal of these compounds for various parameters.

The TNBS test discussed in Section 3.3.2 was used to measure aniline and *o*-anisidine concentrations. The peak wavelength, peak time and extinction coefficient were determined for both compounds and used in quantifying them. The values are shown in Table 4.1. The progression of change in colour intensity with respect to time is shown in Figure 4.1. There is sharp increase in intensity initially which reaches a plateau before it starts to decrease at a slow rate. Based on this observation, 30 min of time for aniline and 50 min of time for *o*-anisidine was considered as optimal time for the test.

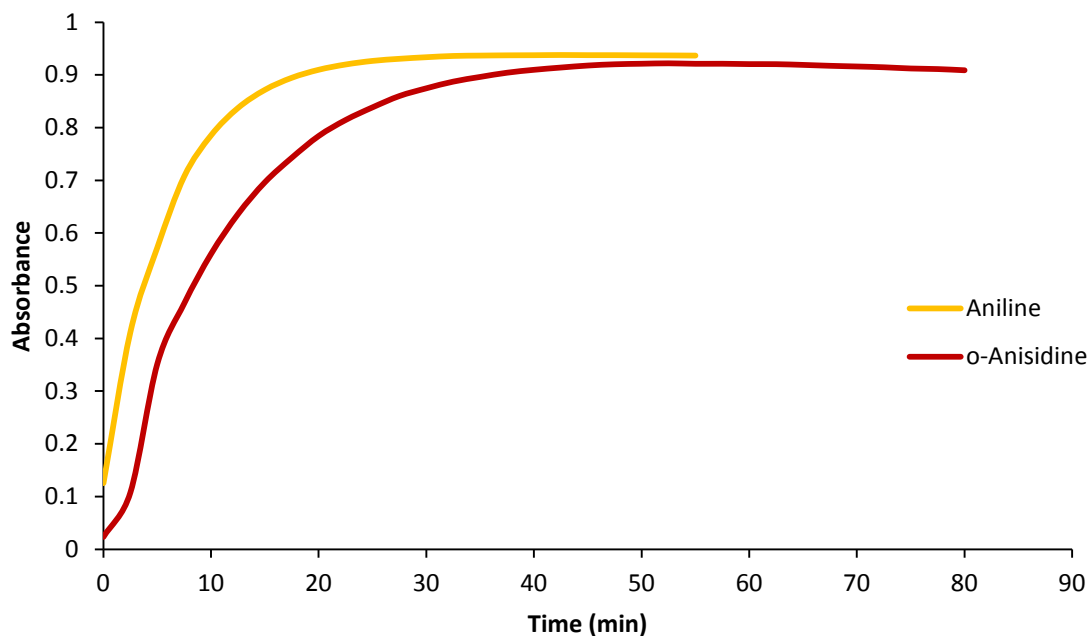


Figure 4.1 Change in colour intensity with respect to time during TNBS test.

Table 4.1 Parameters for analysis of anilines by TNBS test

Substrate	Peak Wavelength (nm)	Peak Time (min)	Extinction coefficient ($M^{-1} \cdot cm^{-1}$)
Aniline	430	30	22300
<i>ortho</i> -Anisidine	430	50	21300

4.1.1 pH Effect

The catalytic activity of a specific enzyme-substrate complex is dependent on pH (Palmer, 1991). This is partly due to changes in characteristics of ionizable side chains of amino acids caused by change in pH. Denaturing of the protein can occur at extreme pH. Furthermore, the degree of ionization of certain amino side chains can effect enzyme activity. For these reasons, it is important to study the effect of pH on enzyme catalyzed anilines removal. SBP is active at a wide range of pH (2.0-10.0) but since the results of this study relate to wastewater treatment, it was decided to choose a pH range of 3.5 - 7.5 for this study. Batch reactors were run for 3 hours with initial substrate concentration of 1 mM, H_2O_2 concentration of 1.5 mM and an enzyme concentration of 0.17 U/mL for aniline and 0.0035 U/mL for *o*-anisidine, respectively. The experiments were designed to test the effect of pH in the presence of limited SBP concentration so that stringent conditions existed to clearly determine pH effect. Figures 4.2 and 4.3 show the results of these experiments for aniline and *o*-anisidine, respectively.

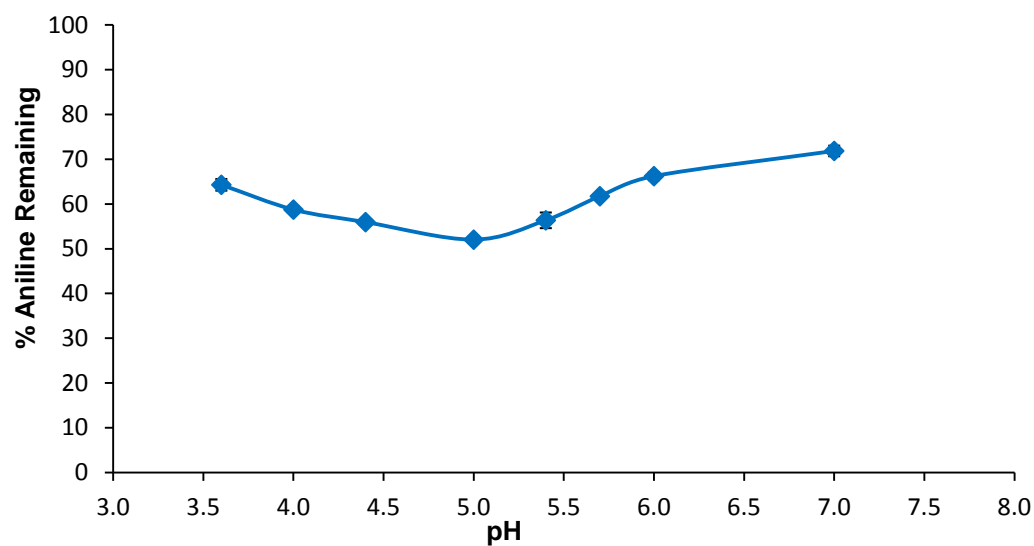


Figure 4.2 Effect of pH on 1 mM aniline removal by 0.17 U/mL SBP with 1.5 mM H₂O₂, reaction time = 3 hours.

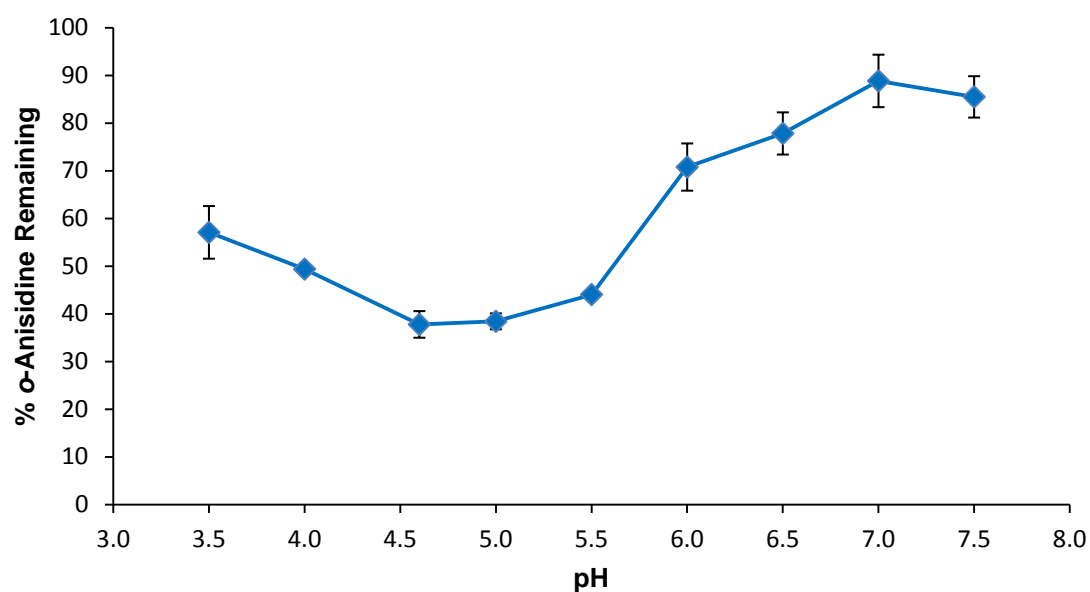


Figure 4.3 Effect of pH on 1mM *o*-anisidine removal by 0.0035 U/mL SBP with 1.5 mM H₂O₂, reaction time = 3 hours.

SBP showed catalytic activity in the full pH range studied (3.5 - 7.5). An optimal pH for aniline removal was at pH 5.0, but acceptable removal, within a 5% difference, was achieved in the pH range of 4.5 to 5.5. The same optimum pH of 5.0 was observed for *o*-anisidine removal as well, with acceptable removal in the range of 4.5 - 5.5. Both of these optimal results are close to the pK_a of the substrate (aniline $pK_a = 4.6$, *o*-anisidine $pK_a = 4.5$) (Mantha 2001; NTP, 2011). The removal of *o*-anisidine was more affected by pH variation than the removal of aniline. There was 20% difference in removal efficiency between optimal pH and the worst-case pH for aniline and 50% difference in the case of *o*-anisidine removal.

4.1.2 SBP Dose

Experiments were conducted for 3 hour duration at the previously established optimal pH of 5.0 by varying SBP dose to determine the optimum SBP dose. All other variables were held constant, H_2O_2 concentration was kept at 1.5 mM, and 1 mM substrate was added to all the samples. The results are shown in Figures 4.4 and 4.5.

Results show 95% removal of aniline at SBP dose of 0.6 U/mL. The concentration of SBP for the removal of 95% of *o*-anisidine was 0.012, which is 1/50 of the enzyme dose required for optimum aniline removal. It was also observed visually in both cases that as the enzyme concentration was increased the colour intensity of the solution also increased, with aniline turning reddish brown and *o*-anisidine a purplish hue. At optimal SBP concentration, precipitate were present in both samples; however, the *o*-anisidine treated sample precipitate were more fine and did not settle as well as the precipitate for aniline treated sample.

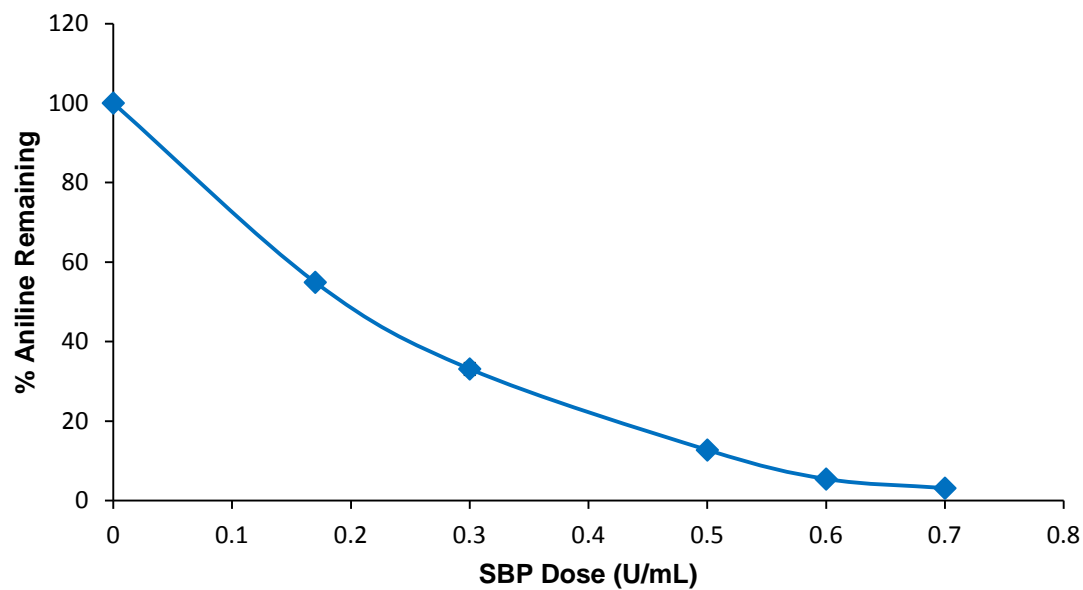


Figure 4.4 SBP optimization for the removal of 1mM aniline, in the presence of 1.5 mM H_2O_2 at pH 5.0, reaction time = 3 hours.

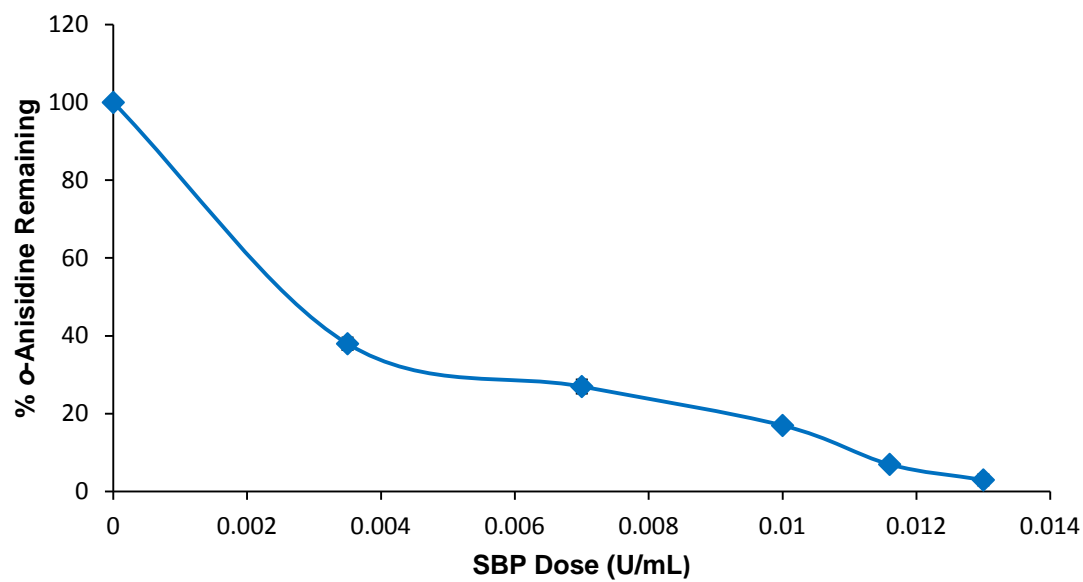


Figure 4.5 SBP optimization for the removal of 1mM *o*-anisidine, in the presence of 1.5 mM H_2O_2 at pH 5.0, reaction time = 3 hours.

4.1.3 H₂O₂ Effect

Peroxidase catalytic reaction stoichiometry, shown by Equation 2.4, indicates a 1:2 molar ratio of H₂O₂ to substrate. However, studies have shown that it is actually closer to 1:1 (Taylor et al., 1998; Ibrahim et al., 2001), while excess H₂O₂ can be limiting (Nicell 1991). For this reason, it was important to check the effect of H₂O₂ concentration on the reaction and how it affected the required SBP concentration. Batch reactors were run for 3 hours with initial substrate concentration of 1 mM, at optimum pH of 5.0 (40 mM acetate buffer) and H₂O₂ was varied for a range of SBP concentrations.

SBP showed catalytic activity in the full range of H₂O₂ concentration studied (0.5 - 3 mM), for both aniline and *o*-anisidine, as seen in Figures 4.6 and 4.7. It is observed that the amount of H₂O₂ required increased with the amount of SBP added and the percent of substrate removed; thus, H₂O₂ demand increased with enzyme activity. At optimal conditions the amount of H₂O₂ required for the treatment of 1 mM aniline with an addition of 0.6 U/mL SBP was 1.5 mM, a 1.5 to 1 H₂O₂ to substrate ratio. In the case of *o*-anisidine, optimal treatment of 1 mM *o*-anisidine was achieved at 1.25 mM H₂O₂, a 1.25 to 1 H₂O₂ to substrate ratio. At this lower H₂O₂ concentration, the amount of SBP needed for 95% removal of *o*-anisidine also was decreased from 0.012 U/mL to 0.0116 U/mL. At higher H₂O₂ concentrations, the percent of substrate removed had decreased, indicating inactivation of SBP by excess H₂O₂. These results are similar to what was found in earlier studies (Arano et al., 1990; Mantha, 2001). The extra H₂O₂ consumption over theoretical stoichiometric requirement is attributed to its consumption by dimeric

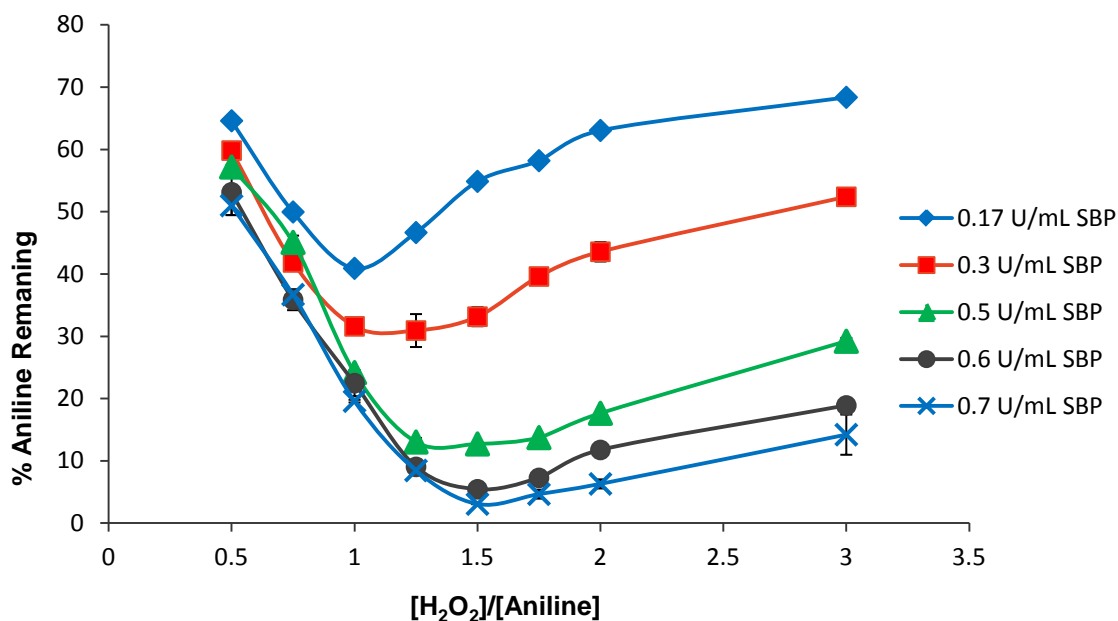


Figure 4.6 Effect of H₂O₂ concentration on the removal of 1.0 mM aniline by SBP at pH 5.0, reaction time = 3 hours.

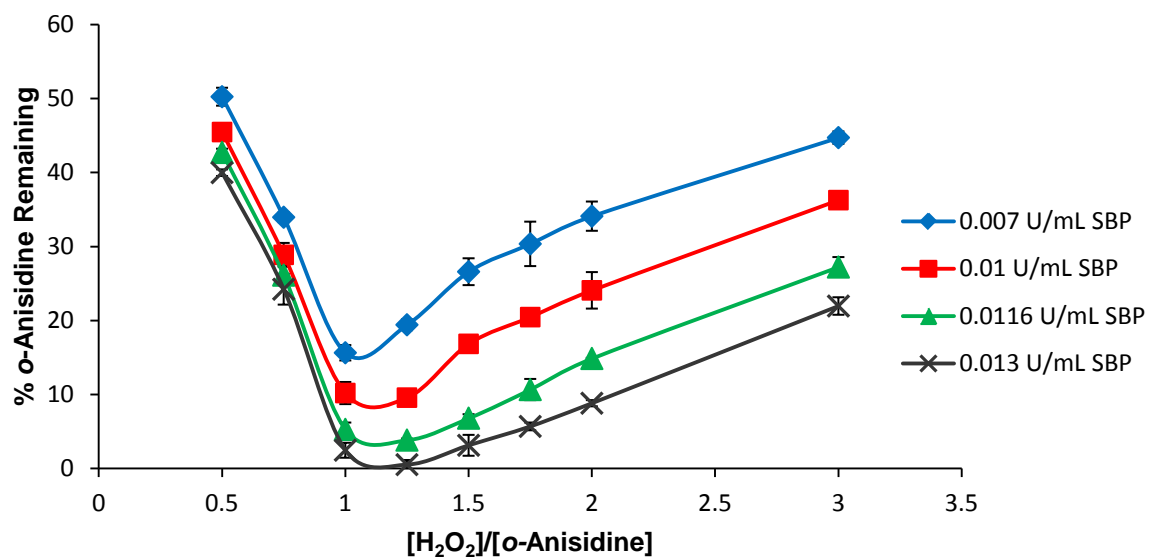


Figure 4.7 Effect of H₂O₂ concentration on the removal of 1.0 mM *o*-anisidine by SBP, at pH 5.0, reaction time = 3 hours.

and polymeric compounds produced in the reaction, as stated by Yu et al. (1994). This had resulted in an overall 1:1 substrate to H_2O_2 ratio. The final optimal parameters for both substrates are included in table 4.2. Crude SBP was noted to have a higher H_2O_2 demand than other peroxidase (Patapas et al., 2007). Biswas (1999) had stated that this increase in demand over a peroxide to substrate ratio of 1 could be a result of catalase activity, which is found in all plants, that can be exhibited by the SBP catalytic reaction. Catalase decomposes hydrogen peroxide to oxygen and water. Another reason for the increase in demand could be a result of hydrogen peroxide oxidation of organic matter present in the mixture.

Table 4.2 Optimal parameters for SBP treatment.

Substrate	pH	[SBP] U/mL	[H_2O_2]:[substrate] (mM:mM)
Aniline	5.0	0.6	1.50:1
<i>ortho</i> -Anisidine	5.0	0.0116	1.25:1

*starting with 1 mM substrate, time = 3 hours.

4.1.4 Effect of Reaction Time

The time needed for a reaction to achieve 95% removal of substrate, otherwise known as retention time in continuous flow system, is an important design factor influencing reactor size, which in turn affects capital cost for implementing enzymatic treatment of industrial wastewater. The kinetic study of substrate removal with respect to time and analysis provides an understanding of enzyme-substrate interaction over the full reaction time. In this study, a 3-hour reaction time was chosen to allow for different parameters and substrates to be compared, thus the goal was to achieve 95% removal in 3 hours.

Results from previous sections 4.1.1 - 4.1.3 were used to set up optimal conditions. In 250 mL batch reactors, 1 mM substrate was treated under optimal conditions and aliquots were withdrawn at various time intervals, quenched with catalase then micro-filtered and analyzed by the TNBS method. Results are shown in Figures 4.8 and 4.9.

SBP's catalytic reaction with both substrates proceeded rapidly at the start, then slowed down after 30 min. Thus, reaction kinetics were looked at in the first 30 minutes in Figures 4.10 and 4.11. It can be seen from the graphs that the reaction with aniline proceeded faster than *o*-anisidine. As a result it is shown that SBP removal of both substrates can be represented as a first-order process, and that the treatment of *o*-anisidine was 25% lower than that of aniline in the first 30 min. Table 4.3 depicts some removal efficiencies as percent of substrate removed with respect to time for both substrates.

Table 4.3 Time for SBP to achieve 50%, 75%, 90% and 95% removal efficiency.

Substrate	Time for Substrate Removal (min)			
	50%	75%	90%	95%
Aniline	10	30	90	180
<i>ortho</i> -Anisidine	20	45	120	180

* Starting with 1 mM substrate at optimum pH, SBP concentration, and H₂O₂ to substrate ratio.

Based on these observations, a 3-hour reaction time was chosen in this study, which provided sufficient time to determine the behaviour of different parameters to achieve 95% removal.

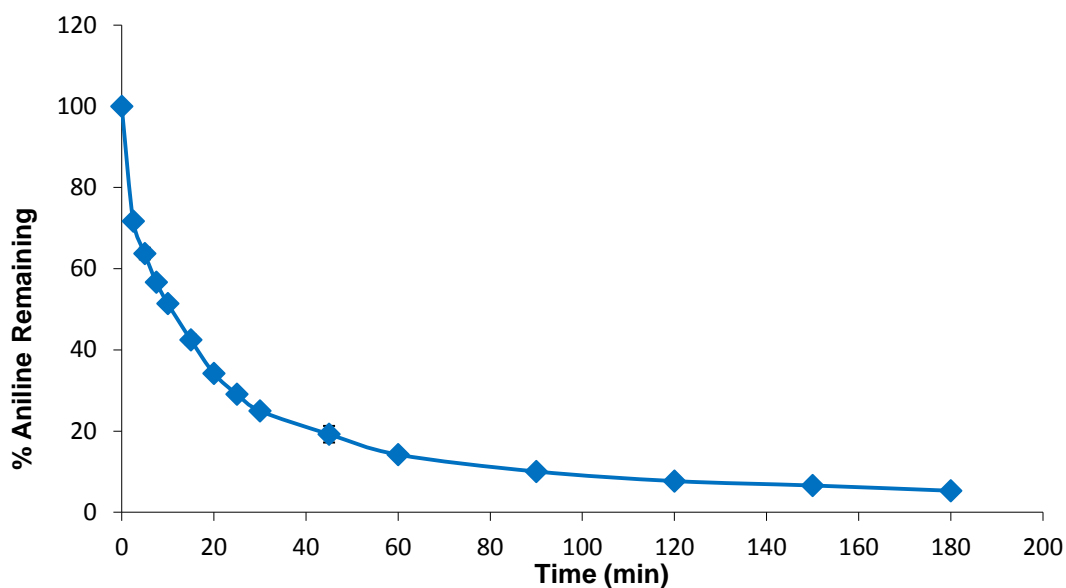


Figure 4.8 Aniline removal at optimal conditions with respect to time. Initially 1 mM substrate, 1.5 mM H_2O_2 and 0.6 U/mL SBP, reaction time =3 hours.

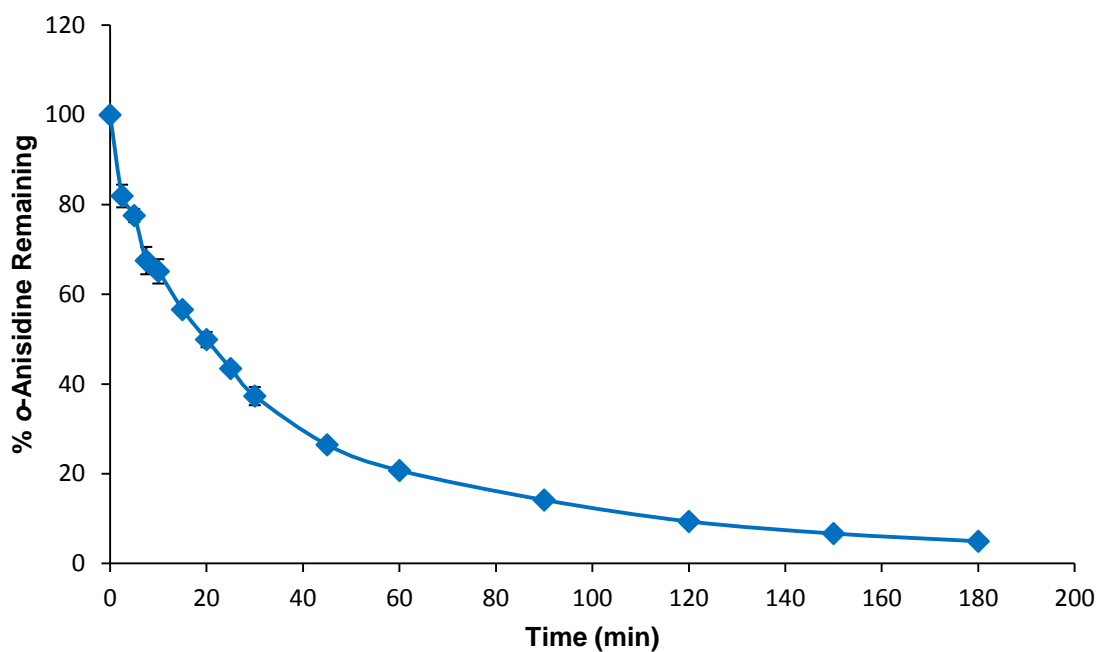


Figure 4.9 *o*-Anisidine removal at optimal conditions with respect to time. Initially 1 mM substrate, 1.5 mM H_2O_2 and 0.6 U/mL SBP, reaction time =3 hours.

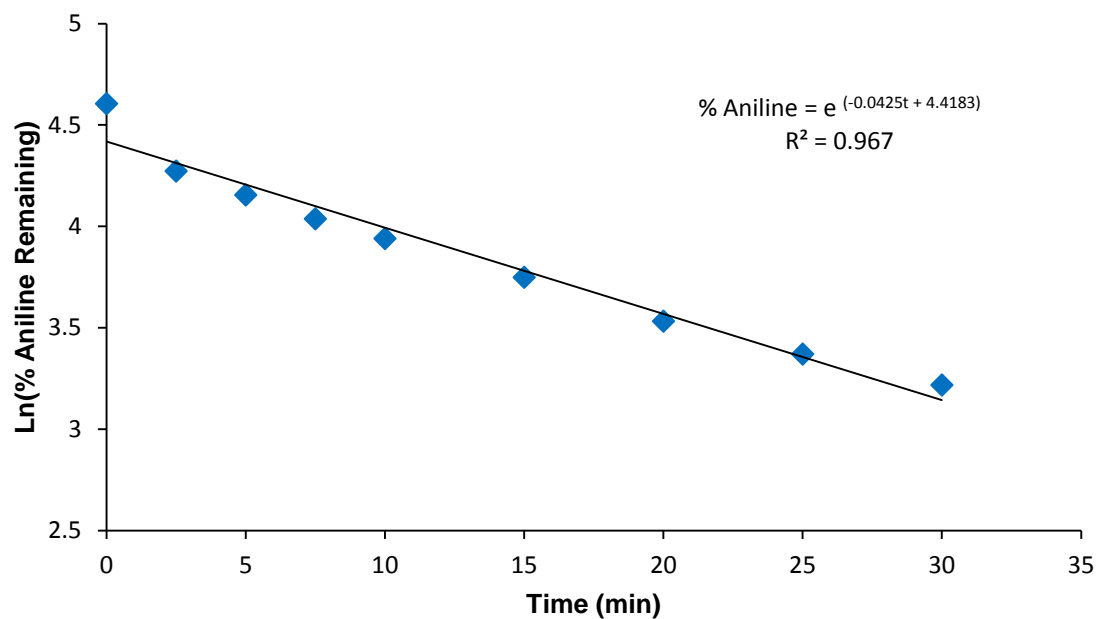


Figure 4.10 Enzymatic treatment of 1.0 mM aniline kinetics

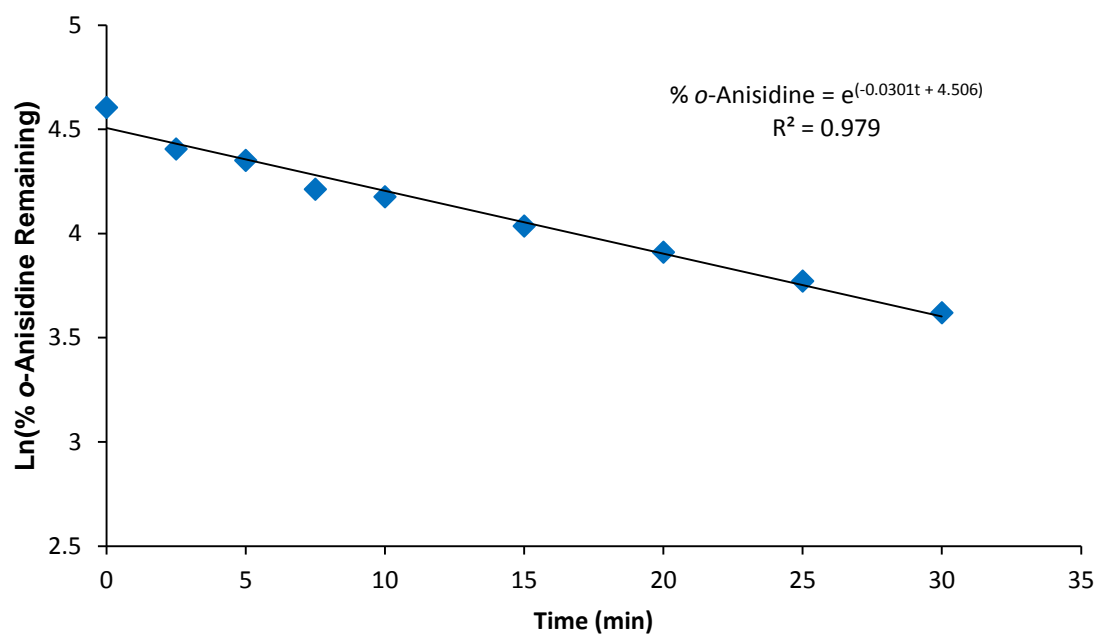


Figure 4.11 Enzymatic treatment of 1.0 mM *o*-anisidine kinetics

4.2 Additive Effect

The effect of various additives on enzymatic treatment of anilines was studied in order to both reduce the enzyme requirement and enhance the effluent quality. However, these additives add carbon to the treatment process and thus have to be accounted for.

4.2.1 Additive Effect on SBP Treatment of Aniline

The use of additives in enzymatic treatment has been shown to be effective in reducing the amount of SBP needed to achieve optimal treatment. Though the use of PEG has shown considerable reduction in the requirement of HRP concentration in the treatment of phenol (Wu et al., 1993), it has not been successful in reducing the amount of SBP needed to achieve optimal removal of the same substrate (Caza et al., 1999). However, other additives such as SDS, and Triton X-100 were shown to effectively decrease the amount of SBP needed in the treatment of phenol (Flock et al., 1999). This experiment was set up to study the effect of different additives on the removal of aniline. The additives chosen included polyetheylene glycol (PEG), a hydrophilic synthetic polymer; sodium dodecyl sulfate (SDS), another anionic surfactant; sodium dodecylbenzenesulfonate (SDBS), an anionic surfactant; Triton X-100, a nonionic surfactant; and sodium dodecanoate (SDOD) an anionic surfactant fatty acid. Batch reactors were set up at pH 5.0 with 1 mM aniline, 0.3 U/mL SBP, and H₂O₂ ranging from 0.5 to 2.25 mM. The concentration of the additives were 100 mg/L except for SDOD where only 50 mg/L was used because of its low solubility. Beyond 100 mg/L, particles can be visibly seen in the reactor. These results are shown in Figure 4.12. It can be seen that PEG has no effect on the SBP treatment of aniline, a result similar to that reported by

Steevensz (2008) in the treatment of aniline with laccase. All other additives showed improvement of enzymatic treatment after their addition, with SDOD having the most significant impact. The only additives that also improved the aesthetic quality of the treated water by removing colour and improving the ability of floc to settle were SDS and SDBS. Figure A.1 in appendix A shows a qualitative comparison of the treated samples.

The effect of additive on optimal pH in enzymatic treatment of aniline was studied by adding Triton X-100 or SDS. Experiments were run under stringent conditions, keeping all parameters constant except for pH. Only 0.2 U/mL SBP was added along with 100 mg/L of additive where applicable. In Figure 4.13, it can be seen that the optimal for the control that had no additive was in the pH range of 4.5 to 5.0 which was also the optimal for the batches that had Triton X-100 or SDS. However, it can be seen that the pH effect is more pronounced with SDS addition when compared to control or Triton X-100.

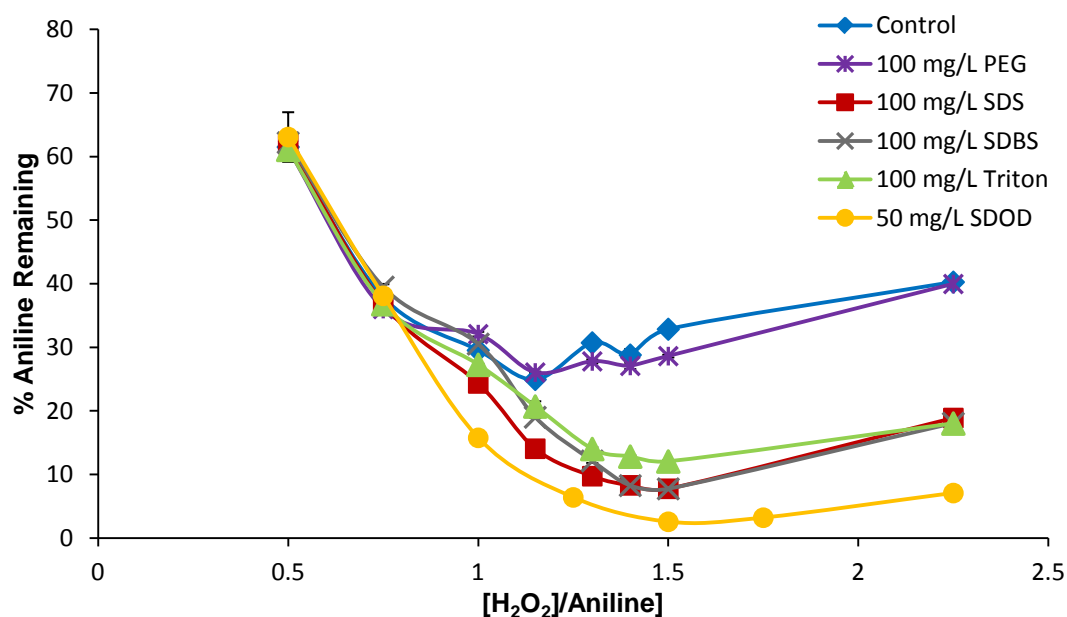


Figure 4.12 Effect of additive addition on treatment of 1 mM aniline with 0.3 U/mL SBP at pH 5.0.

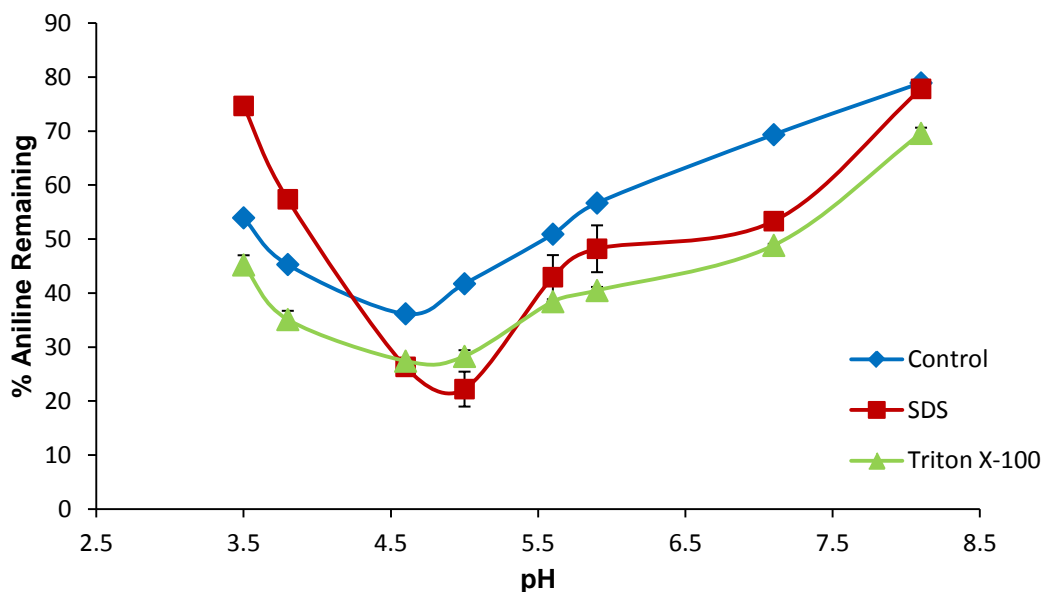


Figure 4.13 Effect of pH change on 1 mM aniline removal with 0.2 U/mL SBP respect to additive addition

The optimal amount of additive required is considered to be the lowest concentration added to achieve the optimal results. Based on the results obtained from the previous experiment, it was decided to only optimize the effect of SDS, Triton X-100 and SDOD. Batch experiments were set up with a starting substrate concentration of 1 mM, at optimal pH 5.0 and 1.5 mM H_2O_2 concentration, and various additives in a range of 0-200 mg/L. SBP was added at 0.3 U/mL to the batch reactors that contained SDS and Triton X-100. Only 0.2 U/mL SBP was added to the samples with SDOD based on the results of previous experiment, Figure 4.12, 0.3 U/mL SBP and 50 mg/L SDOD achieved more than 95% removal. The results showed that 100 mg/L of SDS or Triton X-100 and 50 mg/L of SDOD achieved optimal additive effect as shown in Figure 4.14.

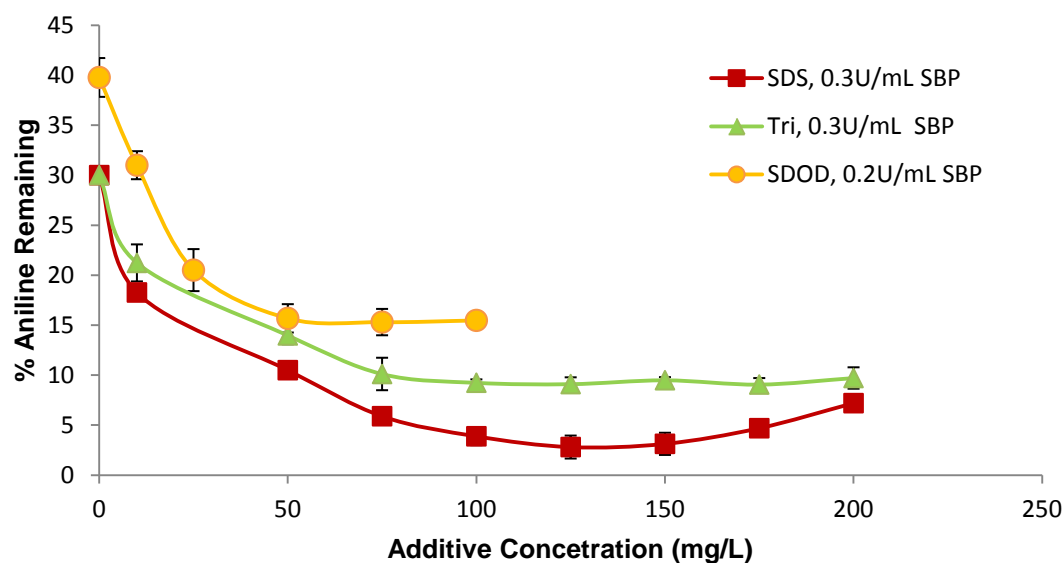


Figure 4.14 Optimization of additive concentration

After having determined that certain additives were able to improve the treatment, it was important to study the effectiveness of these additives on SBP treatment of aniline for better comparison. Experiments were conducted to first find optimal SBP concentration and then optimal H_2O_2 concentration in presence of these additives. To determine optimal SBP, parameters were set at optimal pH 5.0 and H_2O_2 concentration 1.5 mM. The results in Figure 4.15 show the extent of treatment enhancement by additives: with Triton X-100 where only 2/3 of SBP was needed; with SDS addition, the amount of SBP needed was cut by half; with SDOD addition, less than half of SBP was needed when compared to the SBP required in absence of additives. Once the SBP dose values were set, H_2O_2 was optimized. The results shown in Figure 4.16 indicate that, for 95% aniline removal, 1.5 to 1 H_2O_2 to substrate ratio was needed regardless of how much enzyme was reduced. Table 4.4 summarizes these results.

Table 4.4 Additive effect on SBP treatment of aniline

Additive	pH	[SBP] U/mL	[H ₂ O ₂]:[substrate] (mM:mM)
none	5.0	0.6	1.50:1
SDS (100 mg/L)	5.0	0.3	1.50:1
Triton X-100 (100 mg/L)	5.0	0.4	1.50:1
SDOD (50 mg/L)	5.0	0.28	1.50:1

*Starting with 1 mM aniline, and time = 3 hours

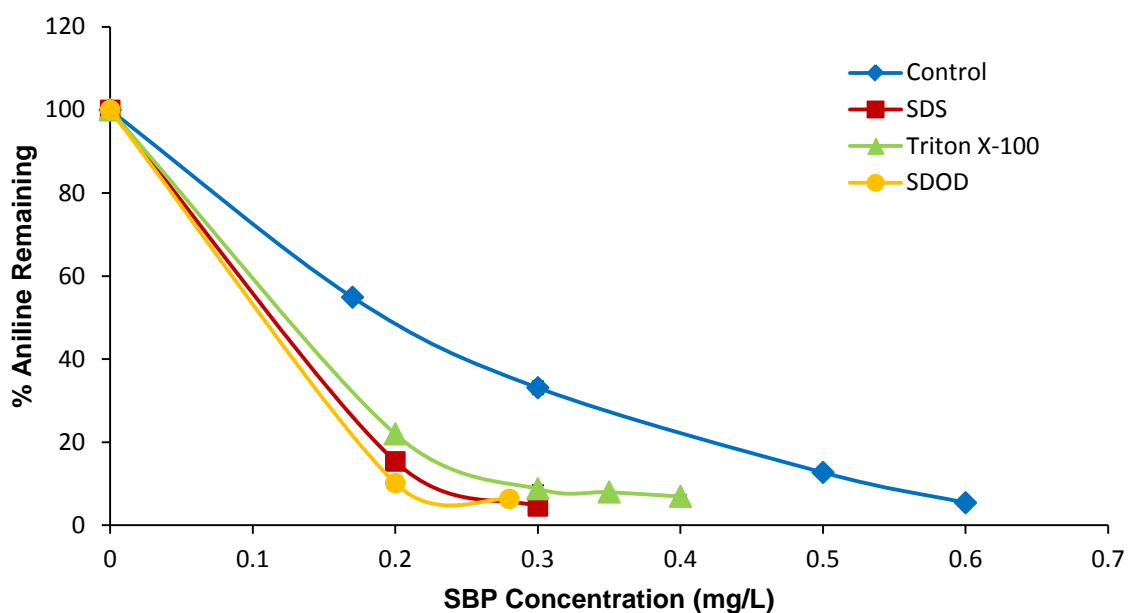


Figure 4.15 SBP dose optimization for 1 mM aniline treatment at pH 5.0. 1.5 mM H₂O₂ for control, SDS and Triton X-100 and 1.25 mM H₂O₂ for SDOD, and reaction time = 3 hours

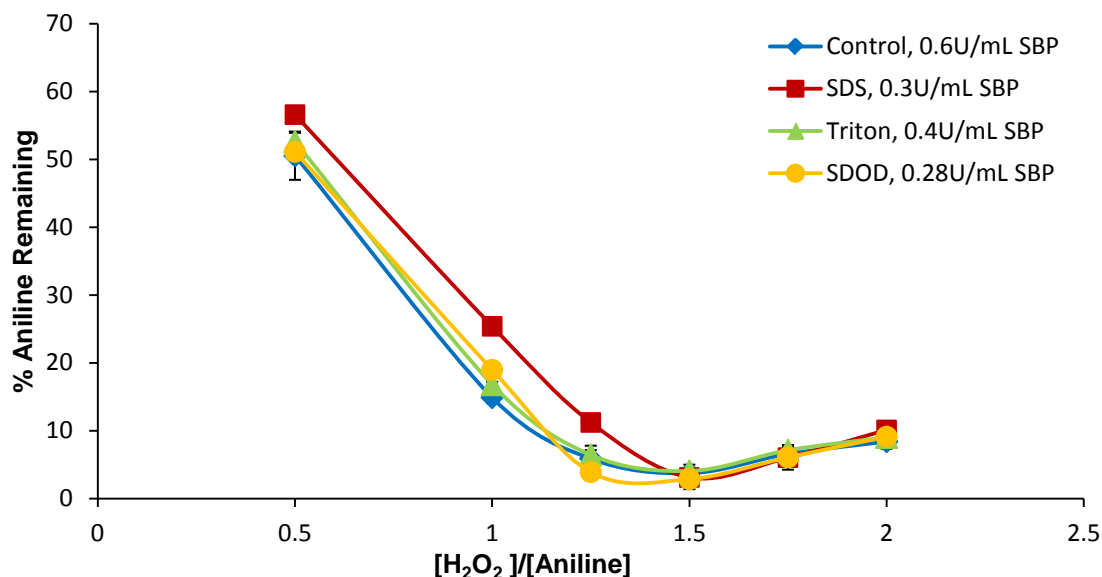


Figure 4.16 H₂O₂ to substrate molar ratio optimization at optimal SBP dose for 1.0 mM aniline, at pH 5.0, and reaction time = 3 hours.

4.2.2 Additive Effect on SBP Treatment of *o*-Anisidine

Since PEG had no effect and SDBS is similar to SDS in both structure and effectiveness, similar experiments were carried out for *o*-anisidine without the use of SDBS and PEG. Batch reactors were set up under stringent conditions at pH 5.0 with 1 mM *o*-anisidine, 1.25 mM H₂O₂, 0.007 U/mL SBP and a range of additive concentrations (0 - 200 mg/L). The results are shown in Figure 4.17. Of the three additives, only SDS significantly improved SBP treatment of *o*-anisidine, starting at 50 mg/L. However, the best colour removal and precipitate formation and settling was achieved with the addition of 100 mg/L of SDS. Based on overall performance, 100 mg/L SDS was chosen as the optimum dose for SDS.

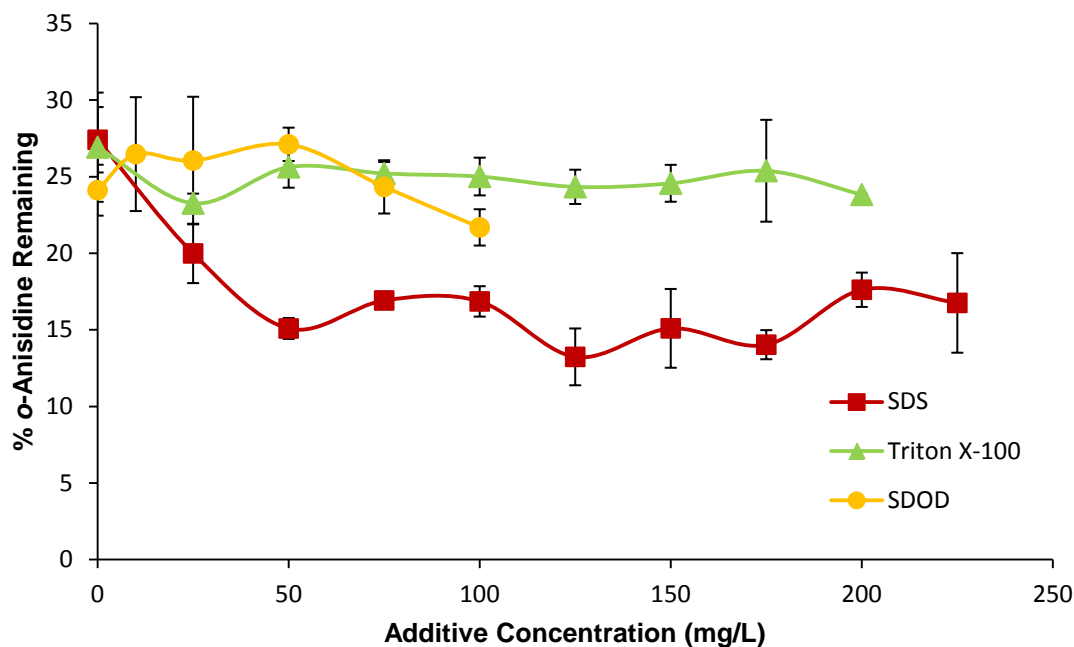


Figure 4.17 Effect of additives on treatment of 1 mM *o*-anisidine with 0.007 U/mL SBP at pH 5.0 with 1.25 mM H₂O₂ and retention time = 3hours.

It was shown in Section 4.2.1 that SDS addition to SBP treatment of aniline was highly dependent on pH. Therefore, experiments were set up to determine optimal pH for the treatment of *o*-anisidine in the presence of 100 mg/L of SDS under stringent conditions. Batch reactors were set up at pH range of 4.0 to 7.5, and all other parameters were kept constant, i.e. H₂O₂ to substrate ratio of 1.25 to 1, and 0.0035 U/L SBP. The results are displayed in Figure 4.18. The optimal pH range was found to be between 6.5 and 7.0, however a pH of 6.0 was chosen as an optimal because it gave better colour removal and precipitate formation.

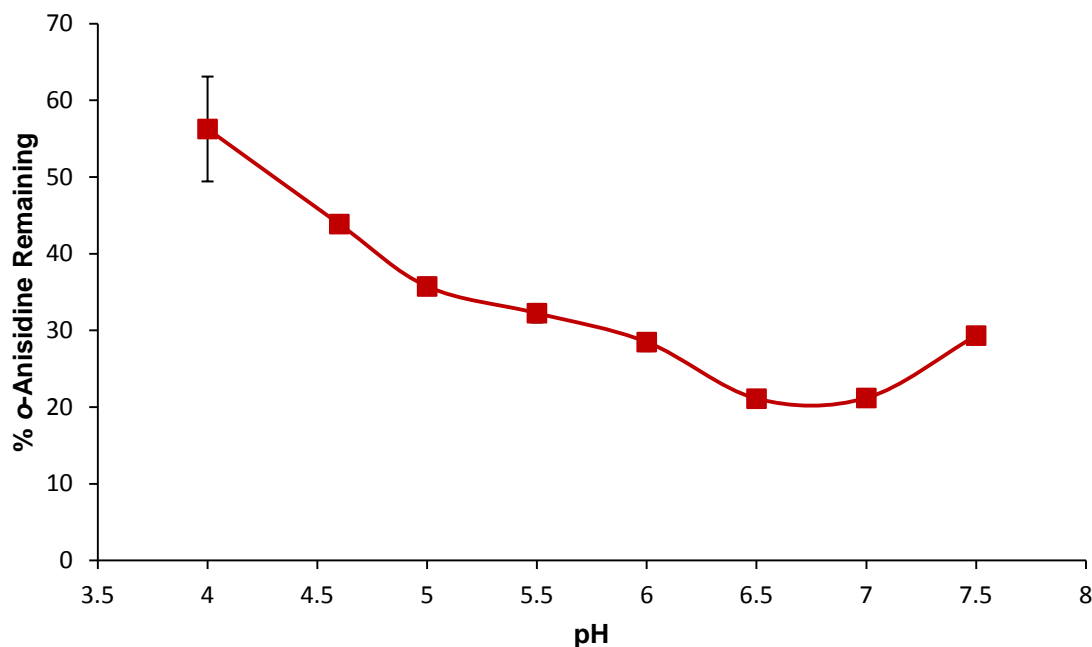


Figure 4.18 pH optimization of treatment of 1 mM *o*-anisidine with 0.0035 U/mL SBP in the presence of 100 mg/L SDS, with 1.25 mM H₂O₂ and reaction time = 3 hours.

Next optimal values for SBP dose and H₂O₂ concentration, needed to achieve 95% removal of *o*-anisidine with the addition of 100 mg/L SDS, were investigated. Batch reactors were set up at various H₂O₂ concentrations, and 0.007 U/mL SBP and run for 3 hours at pH 6.0. The results show (Figure 4.19) that a H₂O₂ to substrate ratio of 1.1 to 1 with SBP dose of 0.007 U/mL achieved >95% removal. This is lower than the ratio of 1.25 to 1 found for *o*-anisidine without additives in Section 4.1.3, Figure 4.7. This result indicates that the increase in H₂O₂:substrate stoichiometry above a 1:1 ratio is related to the crude SBP demand, at lower SBP concentrations the excess demand seems to also decrease. H₂O₂ demand decreased close to 15% as SBP decreased from 0.0116 U/mL to 0.007 U/mL due to additive addition. Table 4.5 summarizes the results for optimal values.

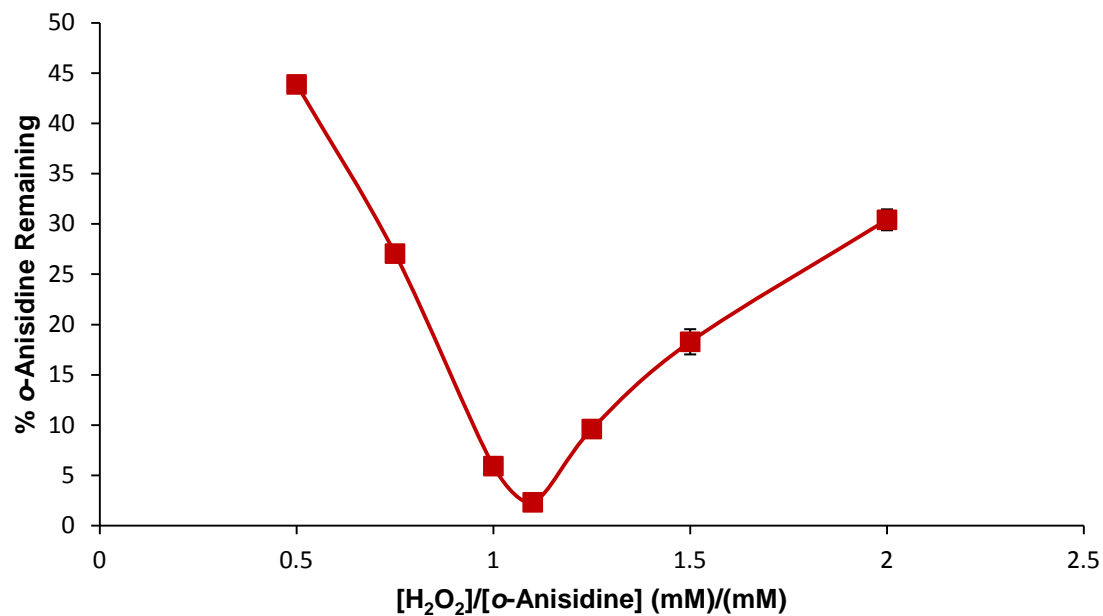


Figure 4.19 Optimization of H₂O₂ at optimal SBP dose of 0.007 U/mL for the treatment of 1.0 mM *o*-anisidine in the presence of SDS.

Table 4.5 Optimal values for additive effect on SBP treatment of *o*-anisidine

Additive	Dose (mg/L)	pH	[SBP] U/mL	[H ₂ O ₂]:[substrate] (mM:mM)
none	0	5.0	0.0116	1.25:1
SDS	100	6.0	0.007	1.10:1

* Starting with 1 mM *o*-anisidine and time = 3 hours

4.2.3 Additive Effect on Reaction Time of Anilines

How additives affect the reaction rate can give insight on how they enhance enzymatic treatment. Batch reactors were set up as in Section 4.1.4 with the optimal amount of additives and experiments were run for 3 hours. The results are displayed in Figures 4.20-4.23. Only SDS was used in the reaction with *o*-anisidine since the other additives had shown no significant effect. Though the presence of additives decreased the amount of SBP required to achieve 95% removal, the time needed to achieve treatment benchmarks of 50%, 75%, and to an extent 90%, had increased as seen in Table 4.6. However, 95% removal was achieved in 3 hours, indicating that, even when the initial rate of reaction had decreased, the reaction rate towards the end was relatively faster. The initial reaction kinetics (first 30 minutes) are shown as first-order plots in Figures 4.24 and 4.25, and the equations of best fit are provided in Table 4.7. It can be seen in Figure 4.24 that the reaction in the presence of SDS has the slowest initial reaction rate. Those reactions in the presence of Triton X-100 and SDOD have a similar reaction kinetics but are still slower than the control. The treatment of *o*-anisidine in the presence of SDS also was slower than that without the additive (Figure 4.25).

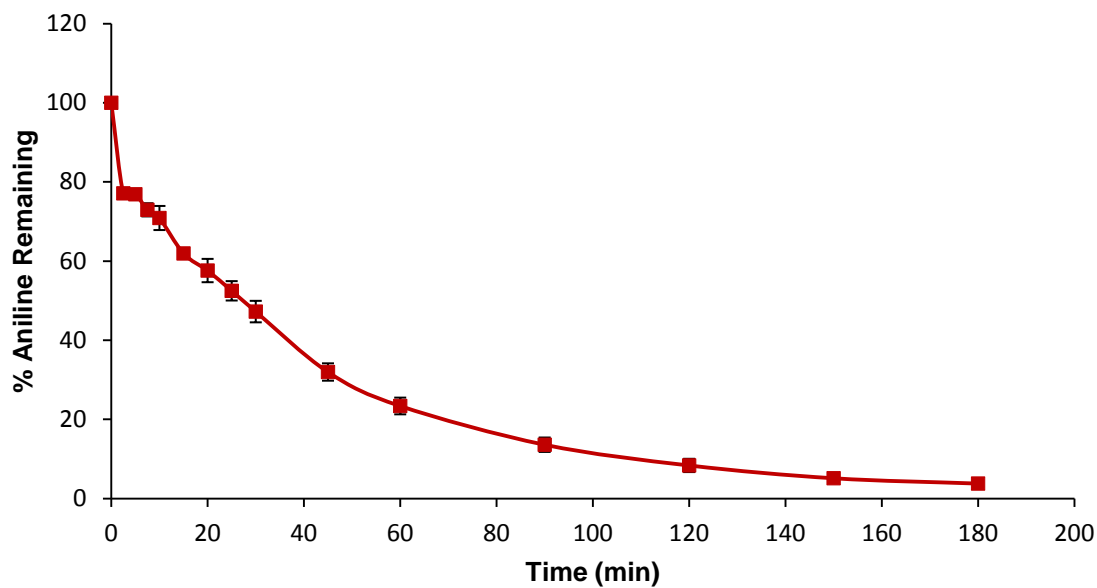


Figure 4.20 Aniline removal by 0.3 U/mL SBP in presence of 100 mg/L SDS with respect to time. Initially 1 mM aniline at pH 5.0 with 1.5 mM H_2O_2 and 3 hour reaction.

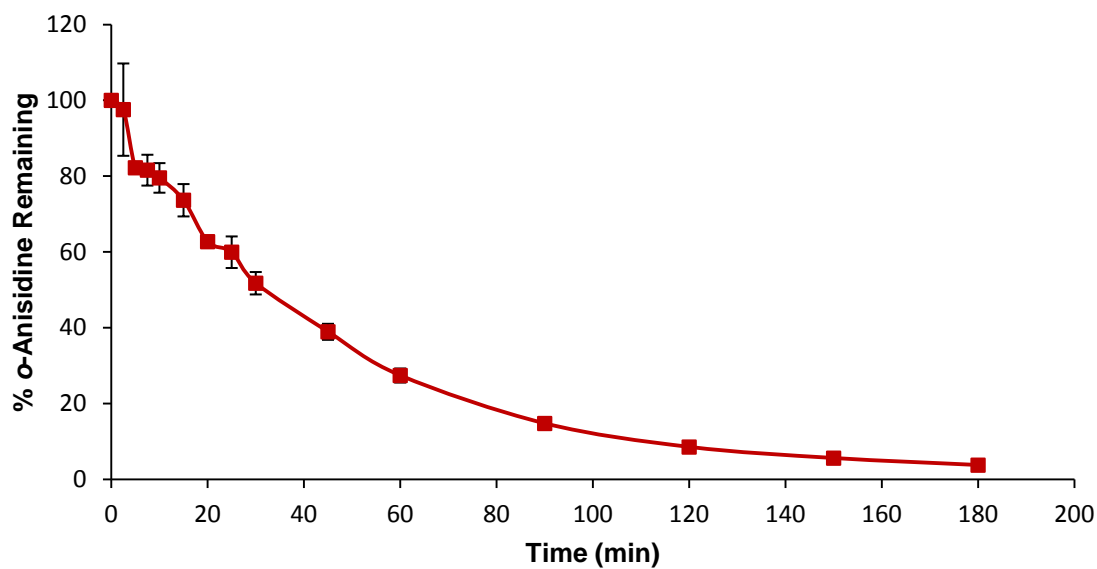


Figure 4.21 *o*-Anisidine removal by 0.007 U/mL SBP in presence of 100 mg/L SDS with respect to time. Initially 1 mM *o*-anisidine at pH 5.0 with 1.1 mM H_2O_2 and 3 hour reaction.

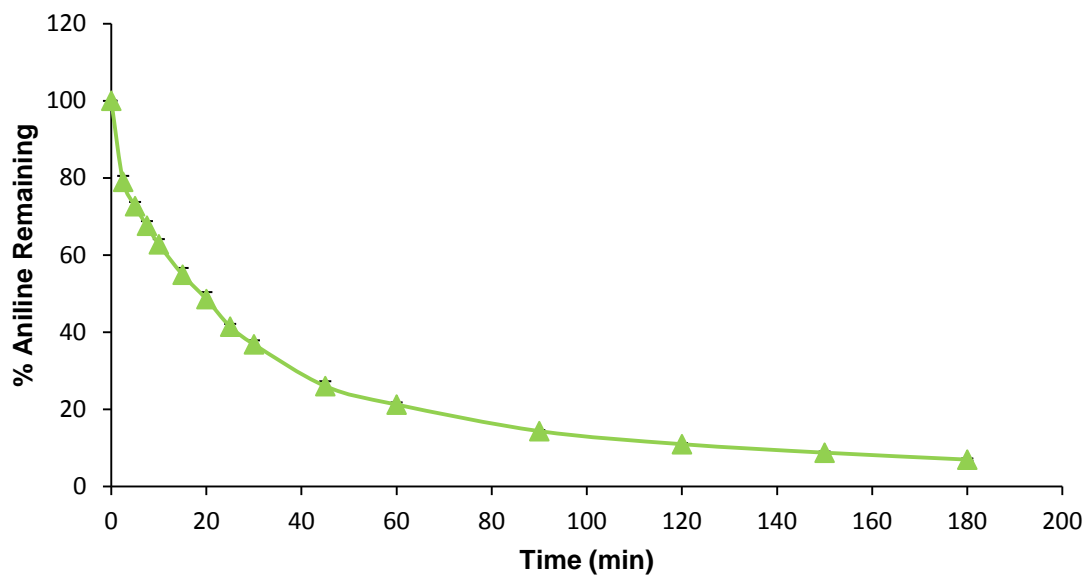


Figure 4.22 Aniline removal by 0.4 U/mL SBP in presence of 100 mg/L Triton X-100 with respect to time. Initially 1 mM aniline at pH 5.0 with 1.5 mM H_2O_2 and 3 hour reaction.

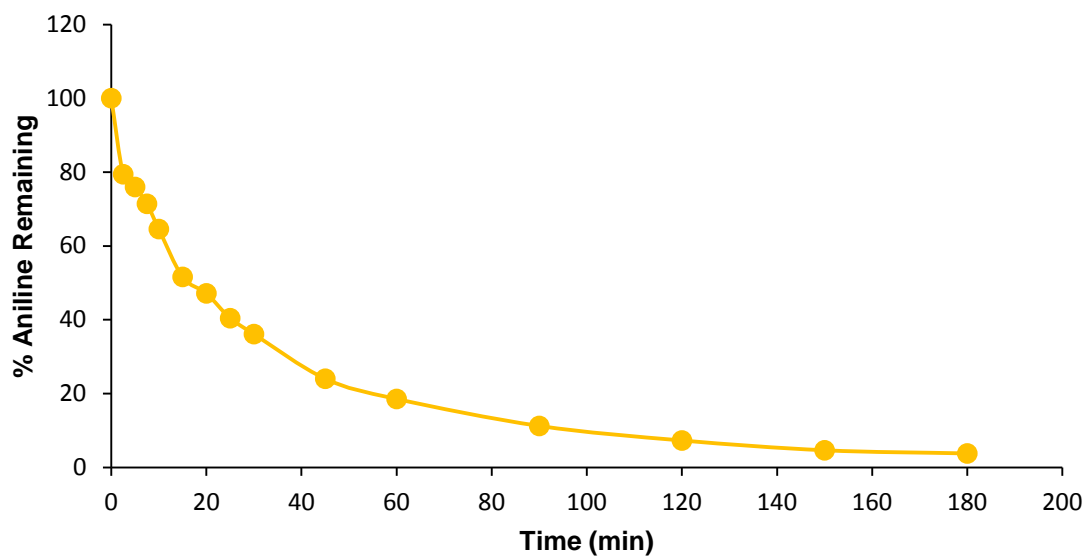


Figure 4.23 Aniline removal by 0.28 U/mL SBP in presence of 50 mg/L SDOD with respect to time. Initially 1 mM aniline at pH 5.0 with 1.5 mM H_2O_2 and 3 hour reaction.

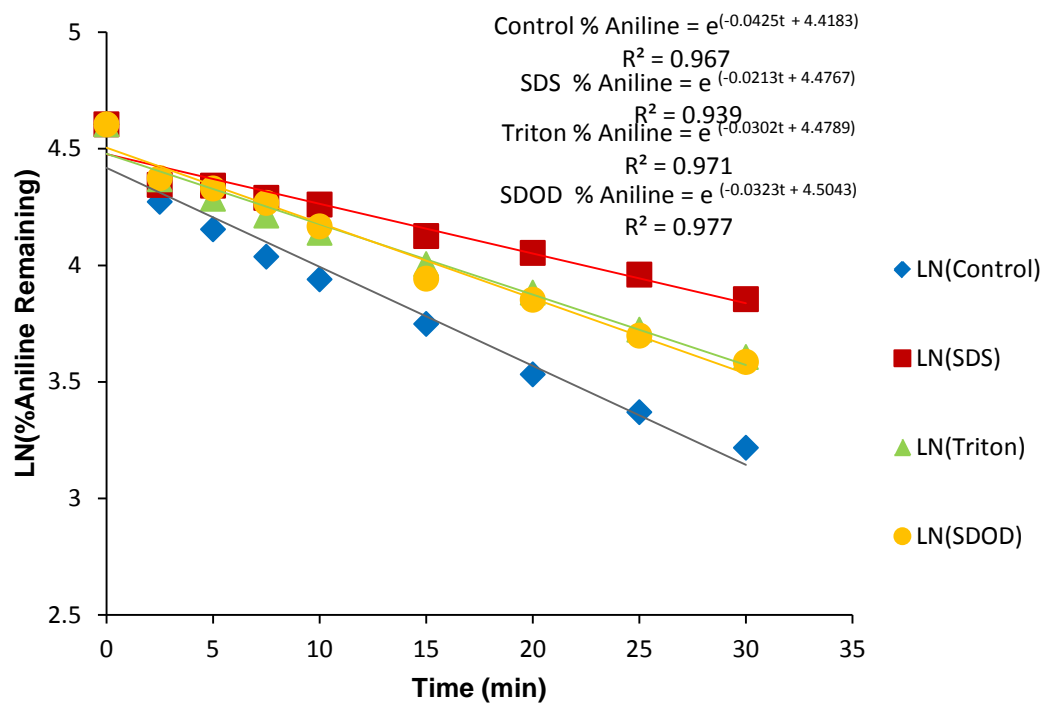


Figure 4.24 Reaction kinetics of enzymatic treatment of 1 mM aniline in the presence of additives at pH 5.0 with 1.5 mM H₂O₂

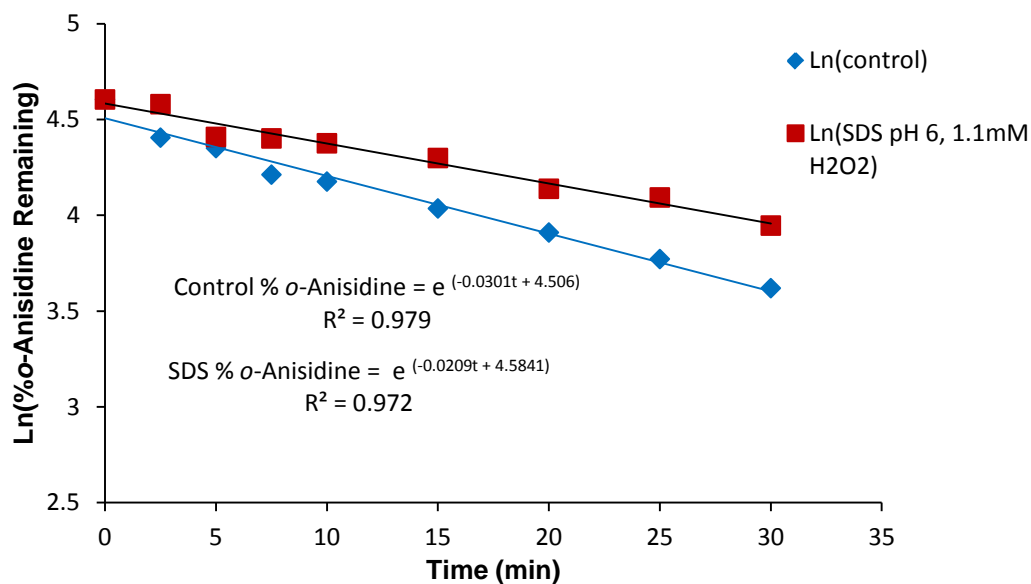


Figure 4.25 Reaction kinetics of enzymatic treatment of 1 mM *o*-anisidine in the presence of 100 mg/L SDS at pH 5.0 with 1.25 mM H₂O₂ for no additive; and 6.0 with 1.1 mM H₂O₂ in the presence of SDS.

Table 4.6 Time for SBP to achieve 50%, 75% and 90% removal efficiency of substrate.

Substrate	Additive Dose (mg/L)	Time for Substrate Removal (min.)		
		50%	75%	90%
Aniline	0	10	30	90
Aniline +SDS	100	25	60	120
Aniline +Triton X-100	100	20	45	120
Aniline + SDOD	50	15	45	90
<i>ortho</i> -Anisidine	0	20	45	120
<i>ortho</i> -Anisidine + SDS	100	30	60	120

* Starting with 1 mM substrate

Table 4.7 Equations of best fit

Substrate	Equation of Best Fit
Aniline	% Aniline = $e^{(-0.0425t + 4.418)}$
Aniline +SDS	% Aniline = $e^{(-0.0213x + 4.477)}$
Aniline +Triton X-100	% Aniline = $e^{(-0.0302x + 4.479)}$
Aniline + SDOD	% Aniline = $e^{(-0.0323x + 4.504)}$
<i>ortho</i> -Anisidine	% <i>o</i> -Anisidine = $e^{(-0.0301t + 4.506)}$
<i>ortho</i> -Anisidine + SDS	% <i>o</i> -Anisidine = $e^{(-0.0209x + 4.584)}$

* Starting with 1 mM substrate and time = 30 minutes at optimal, pH, H₂O₂, and SBP concentration.

Next, experiments were set up to see how addition of SDS to the enzymatic treatment of aniline could change the optimal reaction time. Batch reactors were set up with a range of SDS from 0 to 225 mg/L, 1 mM aniline, 1.5 mM H₂O₂ and 0.2 U/mL SBP and allowed to run for 3 hours. Samples were taken from the reactors, and after stopping the reaction by the addition of catalase, were analyzed for percent aniline remaining. The experiments were continued by stirring up to 6 hours and then the reaction was stopped with the addition of catalase and analyzed. Results shown in Figure 4.26 clearly show that SDS addition improved enzymatic treatment of aniline by increasing the removal efficiency and also increasing the catalytic life of the enzyme. With no SDS addition, an extra 3 hours past the original 3 hour reaction time gave only an additional 3% aniline reduction, while at the optimal dose of SDS of 100 mg/L, an additional 11% of aniline reduction was achieved. This indicates that the addition of SDS protects the enzyme and increases enzyme's catalytic life.

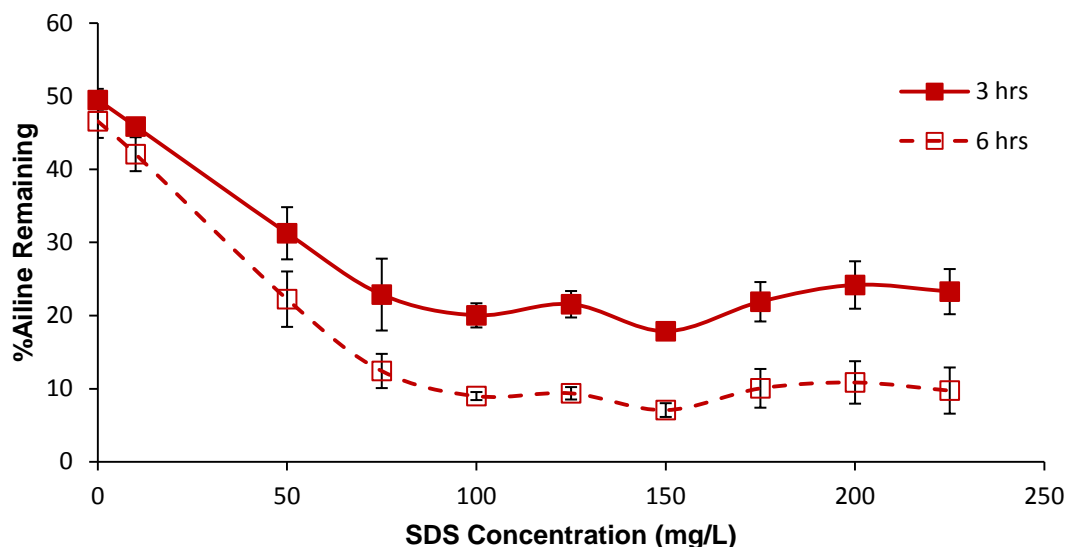


Figure 4.26 Effect of SDS addition on enzyme catalytic life. Initially 1 mM aniline in the presence of 100 mg/L SDS with 1.5 mM H₂O₂, at pH 5.0 and reaction time = 3 hours.

4.2.4 Additive Fate in Treatment - TOC Study

The use of an additive in enzymatic treatment has to take into consideration its possible negative effect on the environment if the excess amount is released with the effluent. The decision to study SDS, Triton X-100 and SDOD as additives was due to their wide use in everyday household detergents. Thus, their presence in the effluent may not cause any toxic effects but a large concentration would have an oxygen demand which may lead to a negative environmental impact. Although, in this study, the additive dose was minimized to achieve maximum results at lowest dose, it was important to study the effect of these additives on the final effluent. Batch reactors were run for 3 hours in non-pH-adjusted tap water with 1 mM substrate, optimal H_2O_2 to substrate ratio, optimal SBP dose and optimal additive dose, (Tables 4.4 and 4.5) and then analyzed for aniline remaining and TOC. Table 4.8 displays the results. Since the reactions were not pH-adjusted, 95% aniline removal was not achieved. The results displayed in Figures 4.27 and 4.28 show the actual TOC values of the samples observed versus the sum of TOC calculated by theoretically adding SBP, additive, and aniline remaining. Experiments in which aniline was the substrate showed that with no additive, the amount of TOC observed was comparable to the calculated amount, indicating that all the enzymatically treated aniline indeed formed polymers that precipitated out of the solution. It can be seen that, with the addition of SDS and SDOD, the amount measured is less than that calculated, indicating that some of the additive might have been removed during the reaction. With Triton X-100 the observed TOC value is slightly above the calculated; however, taking into account the standard deviation, the two values are close enough which allow us to assume that all of the Triton X-100 remained in the solution.

In the treatment of *o*-anisidine with SBP, the amount of TOC observed is higher than that calculated amount for all the samples except the one with SDS. The treatment of *o*-anisidine with SBP produced finer precipitate than those produced during the treatment of aniline. This observation, along with a higher TOC observed value than that calculated value, leads to the assumption that part of the polymers produced remained in solution. The addition of SDS had improved precipitate formation and that may be the reason for the observed TOC to be equal to the calculated TOC. However, this gives no information about the fate of the additive itself.

In all cases, the amount of additive used was below the critical micelle concentration (CMC), 2.33 g/L for SDS and 0.17 g/L for Triton X-100 (Al-Ansari et al., 2010). Respectively, indicating no evidence of partitioning of products into surfactant micelles as a surfactant mechanism which improves substrate removal. More likely the surfactants can resolubilize the active enzyme captured on polymeric precipitate or prevent enzyme adsorption in the first place.

Table 4.8 Percent substrate removal in non-pH-adjusted tap water at optimal parameters

Additive	Dose (mg/L)	% Aniline Remaining	% <i>o</i>-Anisidine Remaining
None	0	15.2	40.9
SDS	100	20.8	1.8
Triton X-100	100	19.8	24.9
SDOD	50	40.4	31.3

* Starting with 1 mM substrate and time = 3 hours in non-pH-adjusted tap water at optimal H₂O₂ and SBP concentration.

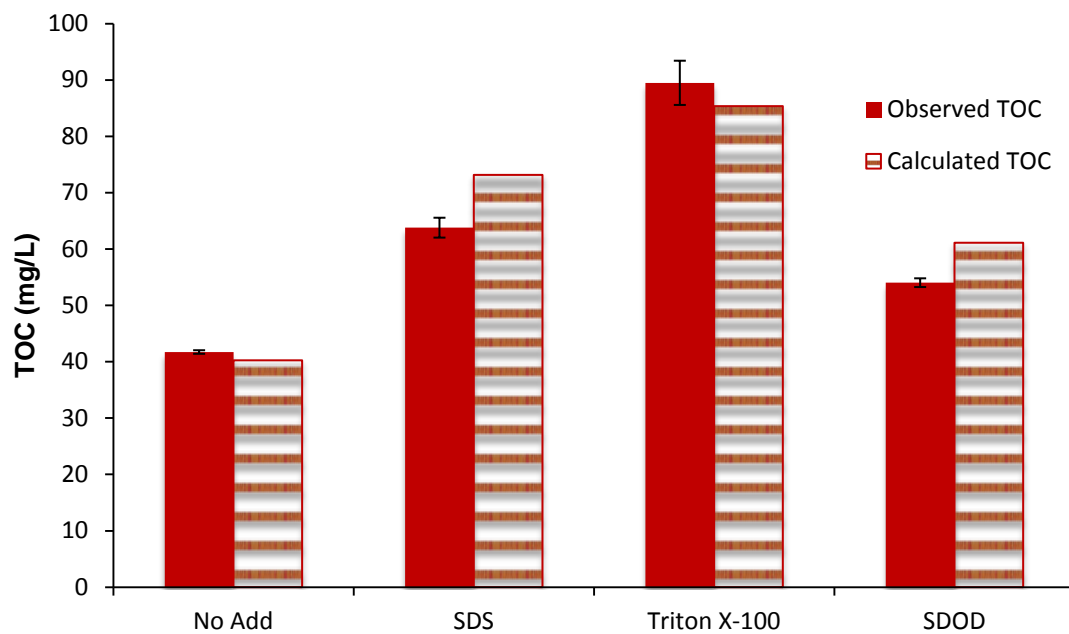


Figure 4.27 TOC of SBP-treated aniline under optimal condition with no buffer

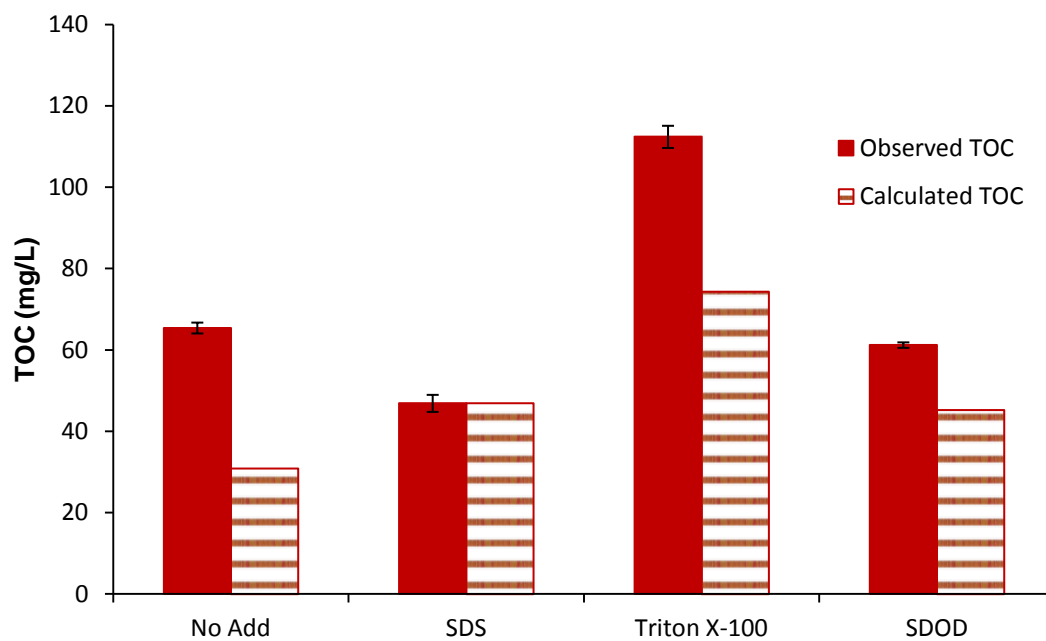


Figure 4.28 TOC of SBP-treated *o*-anisidine under optimal condition with no buffer

4.3 SBP Decolourization of Azo-Dyes

Enzymes have been shown to be effective in directly treating azo dyes (Stibrova et al., 1996; Wang, 2002; Mohan et al., 2005; Knutson et al., 2005). Therefore, the decolourization of COG and AR4 through direct treatment with SBP was studied and optimized. An analytical method was developed, as per Section 3.3.1, to measure the percent colour remaining. A UV-VIS spectrophotometer was used and the results are shown in Figures 4.29 and 4.30. For AR4 maximum absorbance was at $\lambda_{\max} = 508$ nm and for COG it was at $\lambda_{\max} = 482$ nm.

4.3.1 pH Optimization for COG and AR4

As discussed in Section 4.1.1, enzyme activity is dependent on pH. Wang (2002) studied the direct treatment of 50 μ M COG and AR4 with ARP and found the optimal treatment at pH close to neutral pH 7.0 to 8.0. Thus, a slightly wider pH range of 3.5-9.4 than that in Section 4.1.1 was considered for these experiments, to take into account optimal past a pH of 8.0. Batch reactors were set up as per Section 3.4.1 with initial substrate concentration of 1 mM, hydrogen peroxide concentration of 1.5 mM and enzyme concentration of 3 U/mL to ensure reaction. The experiment was carried out for 2 hr. to complete reaction and stopped with the addition of catalase. The results of these experiments are shown in Figure 4.31 for COG and Figure 4.32 for AR4.

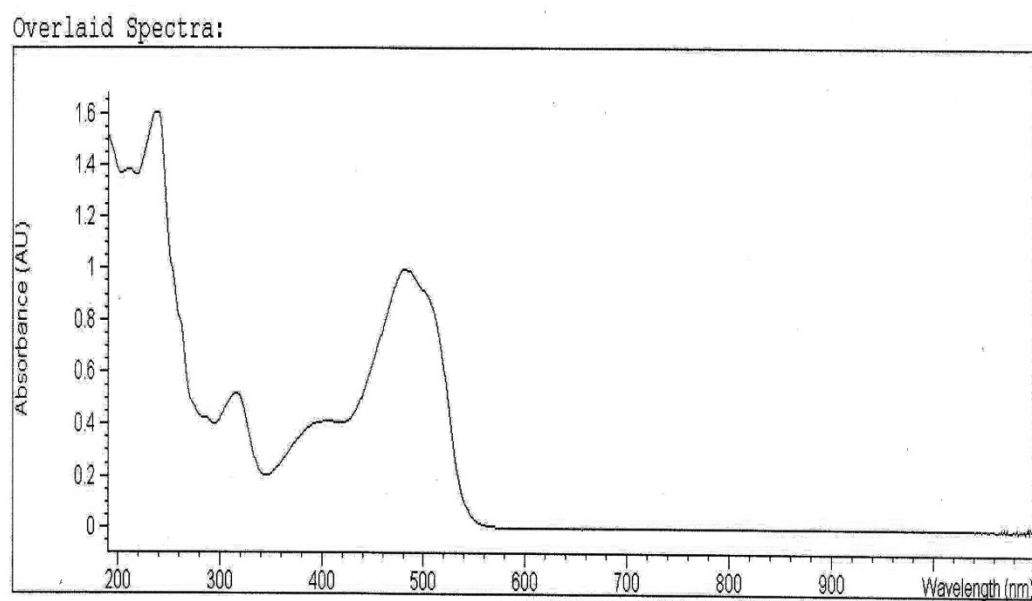


Figure 4.29 UV-VIS spectra for 50 μM COG showing $\lambda_{\text{max}} = 482 \text{ nm}$

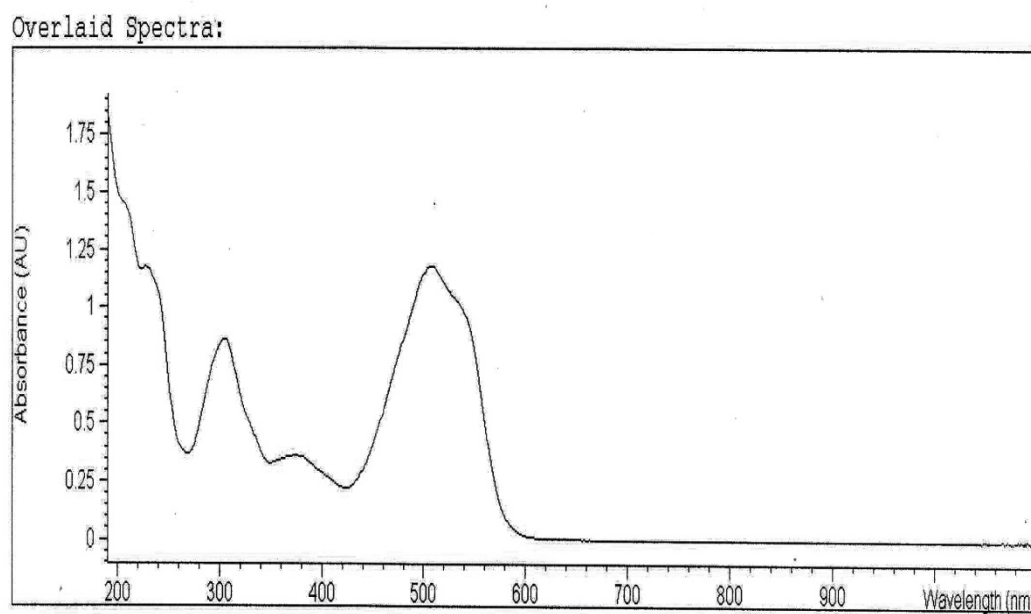


Figure 4.30 UV-VIS spectra for 50 μM AR4 showing $\lambda_{\text{max}} = 508 \text{ nm}$

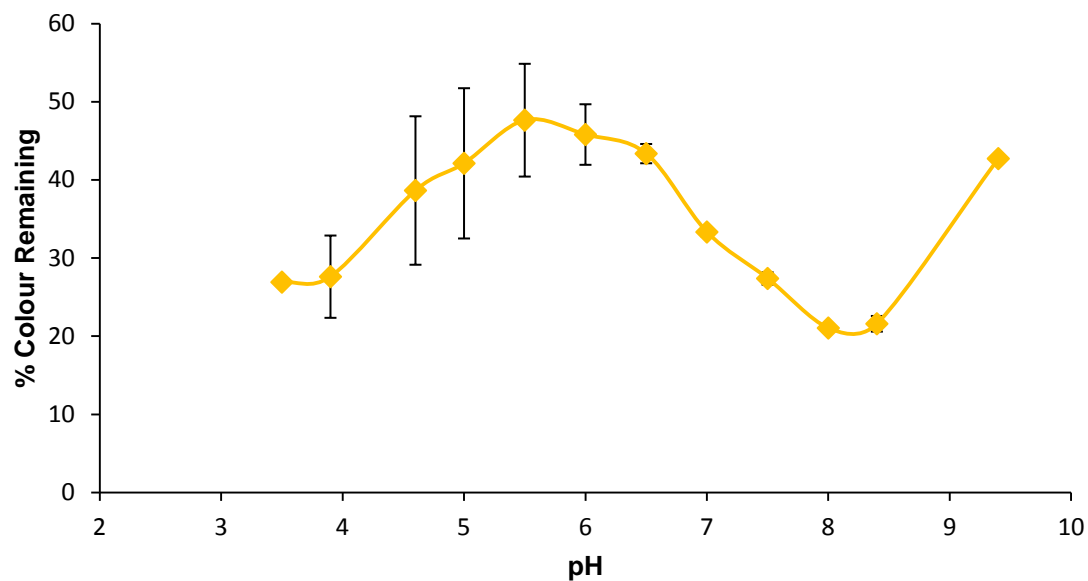


Figure 4.31 pH optimization of SBP-catalyzed COG decolourization. Starting with 1mM COG, 1.5 mM H_2O_2 , 3 U/mL SBP and reaction time = 2 hr.

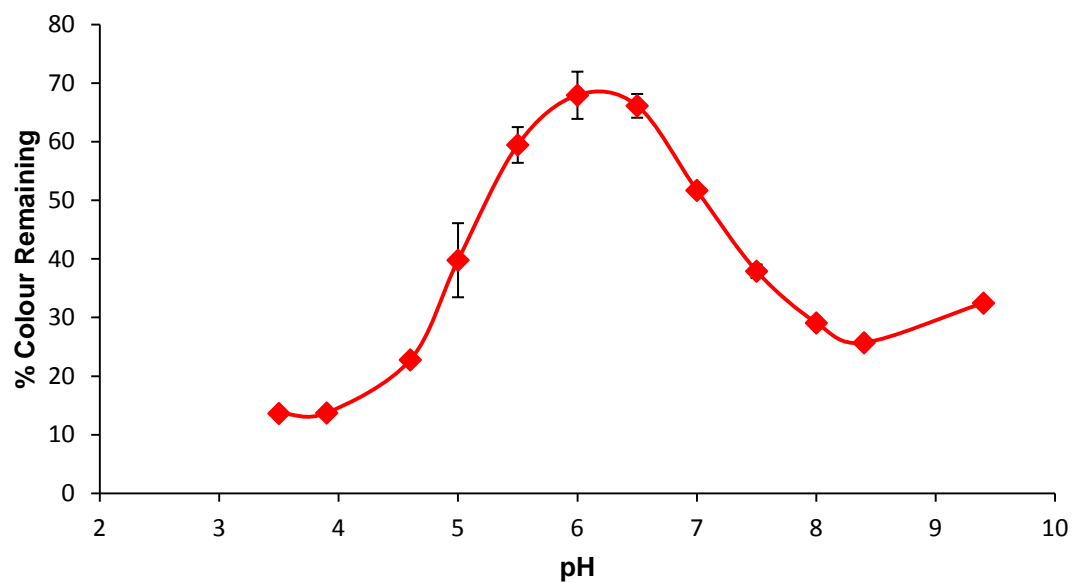


Figure 4.32 pH optimization of SBP-catalyzed AR4 decolourization. Starting with 1mM AR4, 1.5 mM H_2O_2 , 3 U/mL SBP and reaction time = 2 hr.

Both dyes performed well in acidic range of 3.5 - 4.0 and at slightly basic range of 8.0 - 8.5; however, they performed poorly at other pH levels indicating a strong dependence on pH. The optimal pH for COG was in the range of 8.0 to 8.5, however AR4 performed slightly better at the acidic pH range of 3.5 to 4.0. The pH value chosen for both the dyes as an optimal pH for further investigation was 8.0. At this pH value, the effluent was not expected to have any adverse effect on the downstream biological processes, if used, and thus no pH adjustment would be required. A stronger dependence on pH was observed for the treatment of AR4 than treatment of COG. COG showed 27% better decolourization at pH 8.0 than the worst case and at this pH, AR4 colour reduction was 36% better as well.

4.3.2 SBP Dose

The SBP dose for optimal treatment was investigated in this section. Batch reactors were set up at the chosen optimal pH value of 8.0, starting with 1 mM substrate, 3 mM H₂O₂ and SBP dose varied from 0 to 1 U/mL. The reaction was run for 3 hours and results for both optimal SBP dose and optimal colour removal are shown in Figures 4.33 and 4.34.

More than 90% colour reduction of COG was observed with the addition of 0.25 U/mL SBP. An increase of SBP dose to 0.75 U/mL gave 95% colour removal and any increase in the SBP dose beyond that had little effect. In the case of AR4, 75% colour removal was achieved with the addition of 0.25 U/mL. For 84% colour removal an SBP dose of 0.75 U/mL was needed and, as with COG, any increase beyond 0.75 U/mL had only a slight increase in decolourization.

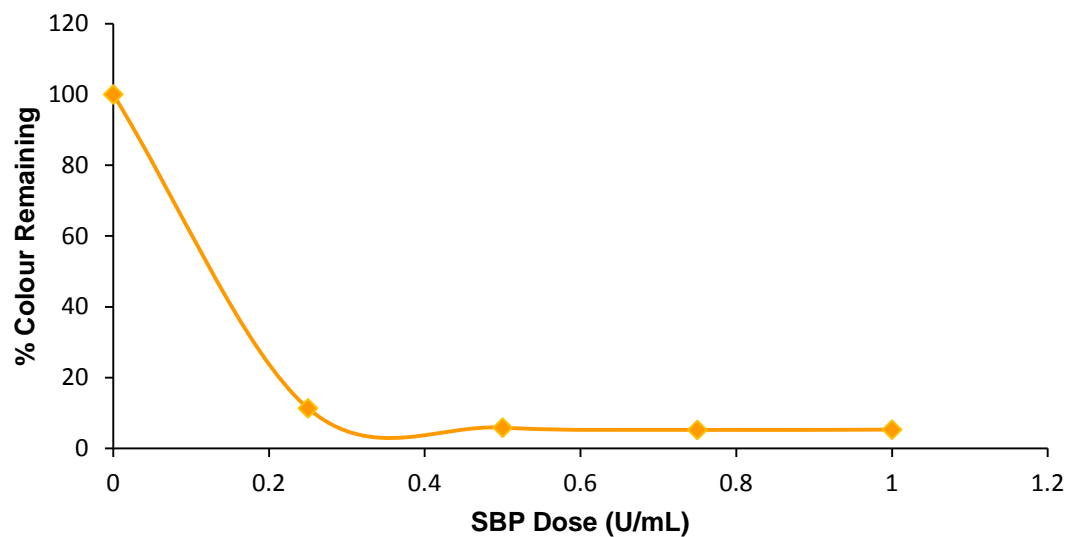


Figure 4.33 Optimization of SBP dose for decolourization of COG. Starting with 1mM COG, 3 mM H₂O₂, at pH 8.0 and reaction time = 3 hr.

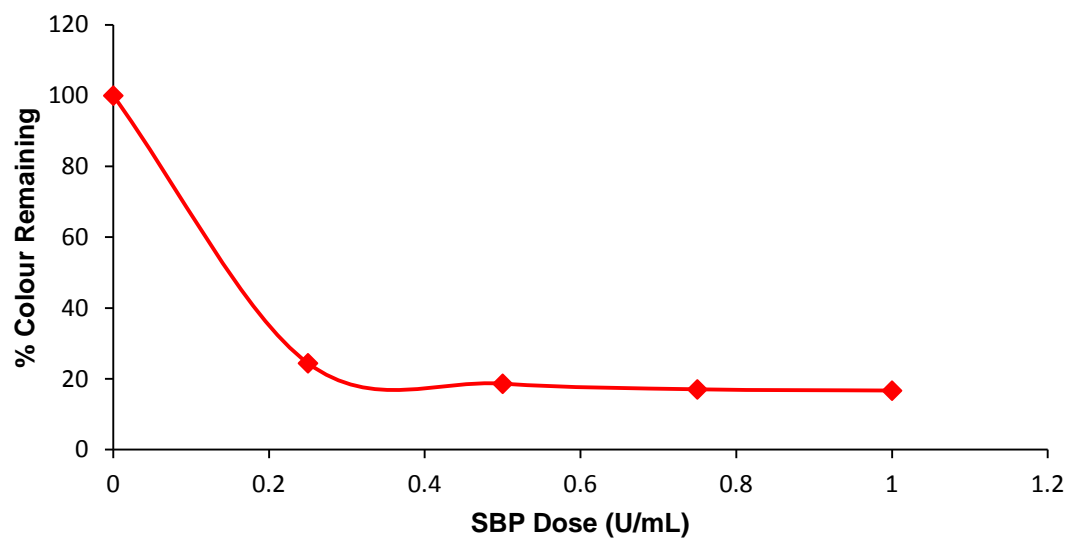


Figure 4.34 Optimization of SBP dose for decolourization of AR4. Starting with 1mM AR4, 3 mM H₂O₂, at pH 8.0 and reaction time = 3 hr.

4.3.2 H₂O₂ Optimization

The effect of initial hydrogen peroxide concentration on colour reduction was studied to determine the optimal concentration. Experiments were conducted with 1mM concentration of azo dye at optimal pH of 8.0. Earlier studies had determined the optimal SBP concentration of 0.75 U/mL and thus various H₂O₂ concentrations were compared at that predetermined optimum value. The results are shown in Figures 4.35 and 4.36. Optimal hydrogen peroxide for 95% colour removal of COG was 3.5 mM for 1 mM COG at optimal SBP concentration. Higher SBP and hydrogen peroxide concentrations were not able to achieve better results. Only 85% colour removal of 1 mM AR4 was achieved with 3 mM H₂O₂ concentration under optimal conditions and, as with COG, higher SBP and hydrogen peroxide concentrations were not able to achieve better results. The lower efficiency of AR4 decolourization could be a result of the meta positioning of the sulfonate group which according to Hsueh et al. (2009) is the least preferred for colour removal, due to lower ability to withdraw electrons from the azo bonds. Another reason could be due to the low purity of the dye, AR4 was 45% pure in comparison to COG which was 90% pure. Some of the residual colour present could be subject to colour artifacts from impurities. These impurities also could have hindered the active site on SBP causing higher enzyme inactivation. The results are summarized in Table 4.9.

Table 4.9 Optimal results summary for SBP treatment of COG and AR4

Substrate	pH	[SBP] U/mL	[H ₂ O ₂]:[substrate] (mM:mM)	Efficiency %
COG	8.0	0.75	3.5:1	95
AR4	8.0	0.75	3.5:1	85

* Starting with 1 mM azo-dye and time = 3 hours.

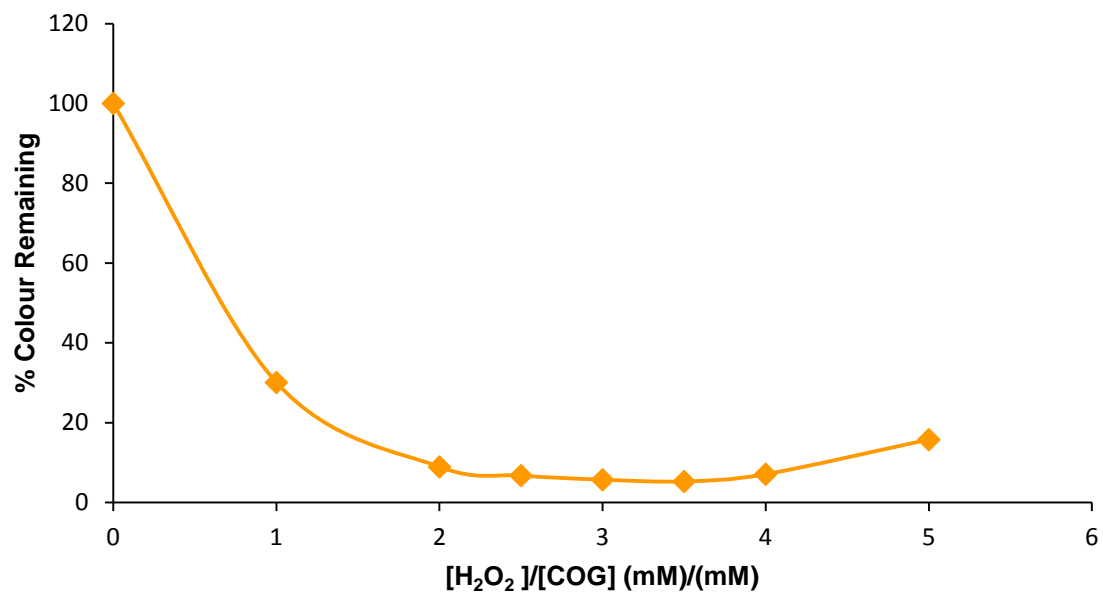


Figure 4.35 Optimization of H₂O₂ concentration for decolourization of 1.0 mM COG, at pH 8.0 with 0.75 U/mL SBP and reaction time = 3 hr.

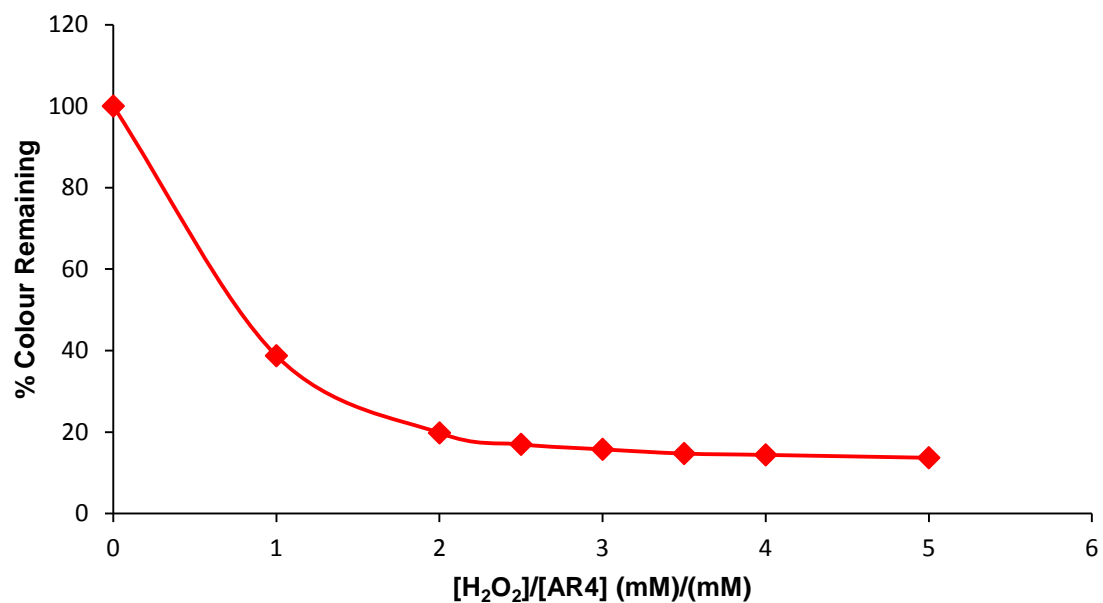


Figure 4.36 Optimization of H₂O₂ concentration for decolourization of 1.0 mM AR4 at pH 8.0 with 0.75 U/mL SBP and reaction time = 3 hr.

4.4 Zero-Valent Iron Reduction Pretreatment of COG & AR4

Zero-valent iron reduction of azo dyes was shown to be an effective method in reducing colour in wastewaters in many studies (Nam and Tratnyek, 1999; Biswas et al., 2007). In Section 4.3, SBP treatment of both COG and AR4 was successful in decolourizing the dyes. The inability of SBP to achieve 95% colour removal of AR4 prompted an investigation into its pretreatment with zero-valent iron. In this section, both COG and AR4, were first pretreated with iron and later their aniline by-products were treated enzymatically.

4.4.1 Optimizing Iron Dosage and Oxygen Scavenger (Sodium Sulfite)

Biswas et al. (2004) had reported that the amount of iron added to the reaction affected decolourization of the dyes. Thus, experiments were set up to study the effect of iron and determine its optimum dose for both dyes. As per Section 3.4.4, batch reactors were run for 30 min with tap water containing 1 mM dye, 1 mM sodium sulfite, and iron ranging from 0 - 2 g. Colour reduction was analyzed by UV-VIS spectrophotometer and anilines production was determined by the TNBS test. The results, shown in Figure 4.37, indicate that the percent colour reduction was proportional to the percent aniline production. The optimal iron dose for COG decolourization was 1 g iron, and an increase to 2 g iron only improved colour reduction by another 6%. However, an increase of iron dose from 1 g to 2 g in the treatment of AR4 improved decolourization by 16%, which indicated an optimal iron dose of 2 g. These results could be due to the lower purity of AR4 which could have hindered iron active sites.

It was suggested by Mantha (2001), while reviewing the literature (Babbitt et al., 1975; Brandvold, 1975), that sodium sulfite, along with cobaltous chloride as a catalyst at 0.1% of sodium sulfite by mass, was an effective oxygen scavenger. Biswas et al. (2004) increased the amount of sodium sulfite proportionally with an increase in iron. Since AR4 was more effectively decolourized with the addition of 2 g of iron, an experiment was set up to investigate the effect of sodium sulfite concentration versus iron dose after 1 hour reaction time. Figure 4.38 shows that 2g iron with 1 mM sodium sulfite provide optimal zero-valent iron decolourization of AR4 and an increase of sodium sulfite to 2 mM showed no effect. This is expected as sodium sulfite scavenges oxygen from water and thus its concentration should be independent of iron dose.

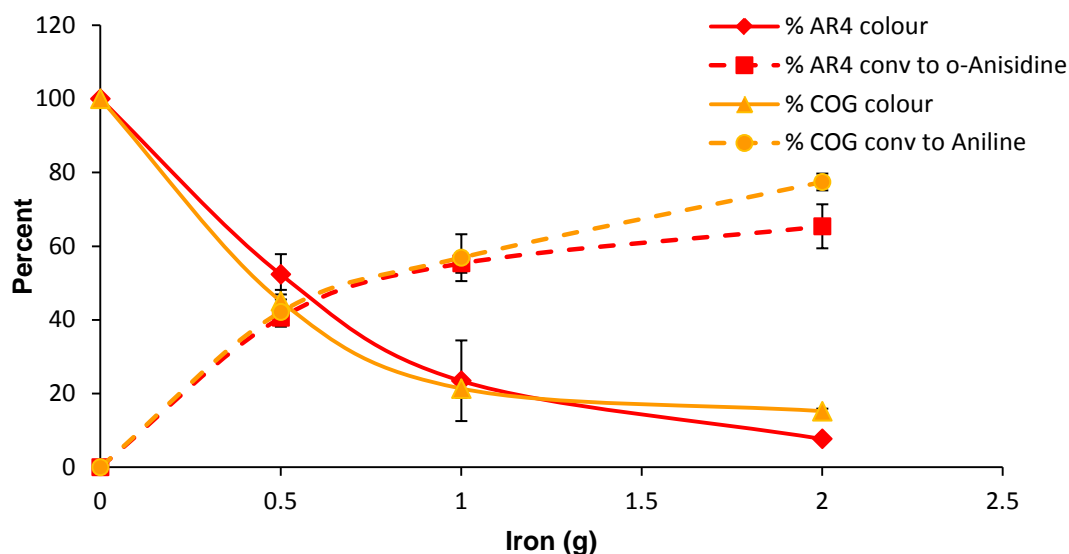


Figure 4.37 Optimal iron dosage for iron reduction of 1 mM azo dyes and production of anilines in presence of 1 mM sodium sulfite and reaction time = 30 min.

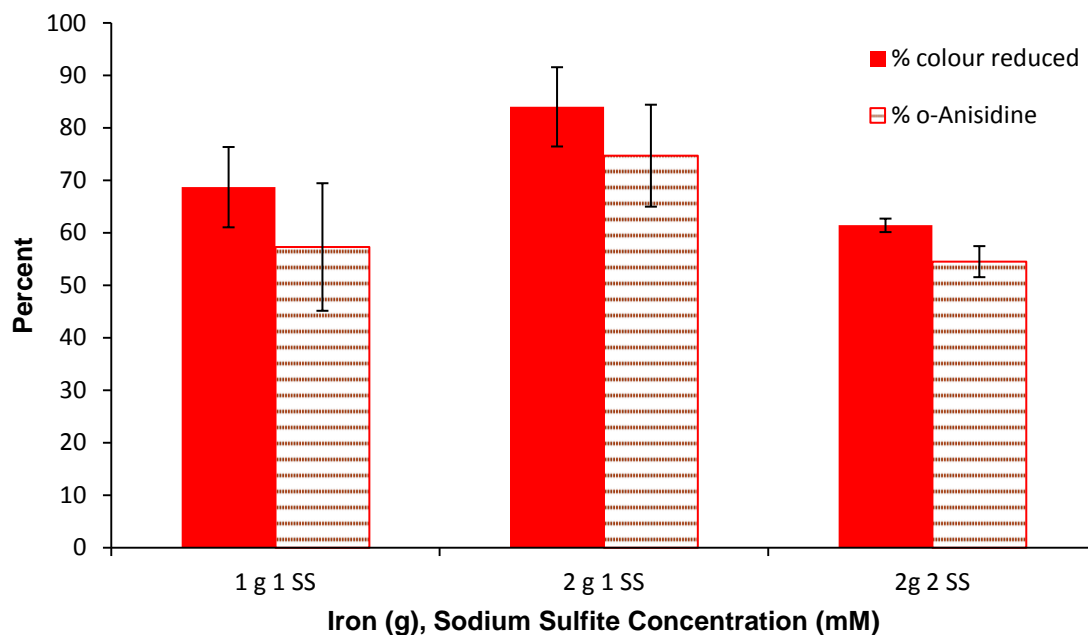


Figure 4.38 Optimization of iron dosage versus sodium sulfite concentration for 1 mM AR4 colour reduction. Reaction time = 1 hr.

4.4.2 Effect of Reaction Time

The time needed to achieve optimal colour removal and anilines production through pretreatment with iron, at the predetermined optimal iron and sodium sulfite doses, was investigated in this section. Batch reactors were set up with reaction time ranging from 0 to 2 hours and samples were analyzed for both colour reduction and product recovery. The reaction time that yielded the highest colour reduction along with the highest product recovery was considered as the optimal time. The results for COG and AR4 are displayed in Figures 4.39 and 4.40.

The optimal time for the treatment of COG is shown to be 75 min at which close to 98% of the colour was removed and 81% of aniline was recovered. The optimal time for the treatment of AR4 was 90 min at which over 96% of colour was removed and 93% of *o*-anisidine was recovered. About 20% of aniline was not recovered in the treatment of COG; however, a better product recovery can be seen for AR4 treatment when about 4 % of *o*-anisidine was not recovered. Mantha (2001) showed that pH affected the reaction, with an acidic pH improving iron reduction and a basic pH giving a better product recovery. For this reason, the pH of the tap water used was not adjusted as changing to either a higher or lower pH could either hinder colour reduction or aniline desorption. Specifically, it was noted that at pH below the pK_a none of the aniline would be desorbed from the iron, leading to a need for extra treatment. These results indicate that iron pretreatment of AR4 was more effective than that of COG. Table 4.10 summarizes the results.

Table 4.10 Summary of optimal results for zero-valent iron reduction of COG and AR4

Substrate	Iron (g)	Sodium Sulfite Conc. (mM)	Time (min)	% Colour Removed	% Product Recovered
COG	1	1	75	98	81
AR4	2	1	90	96	93

* Starting with 1 mM azo-dye

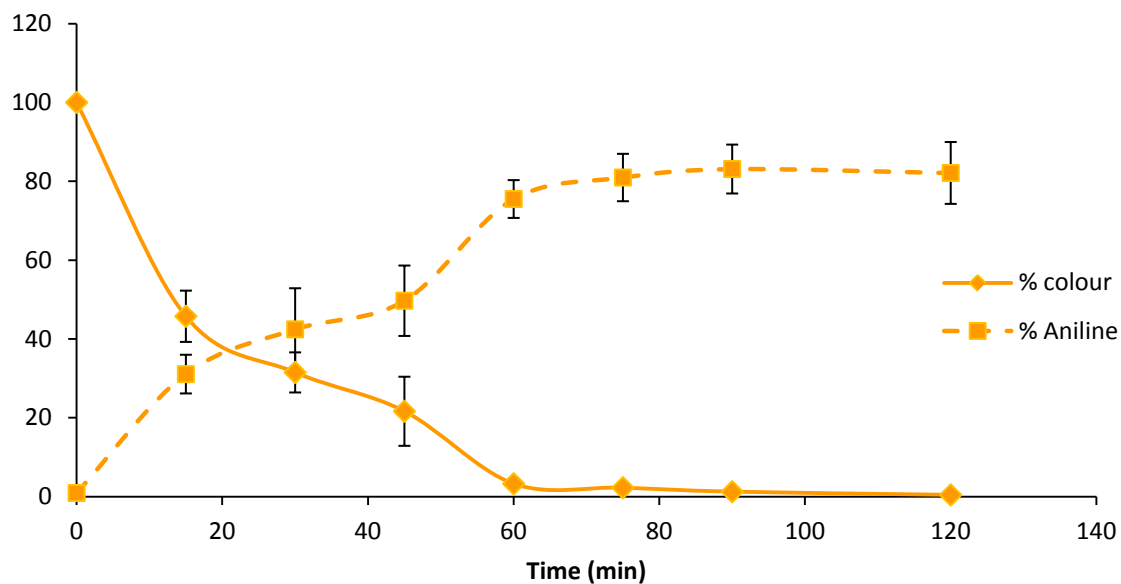


Figure 4.39 Progressive COG colour reduction and aniline production with time when treated with 1 g iron and 1 mM sodium sulfite.

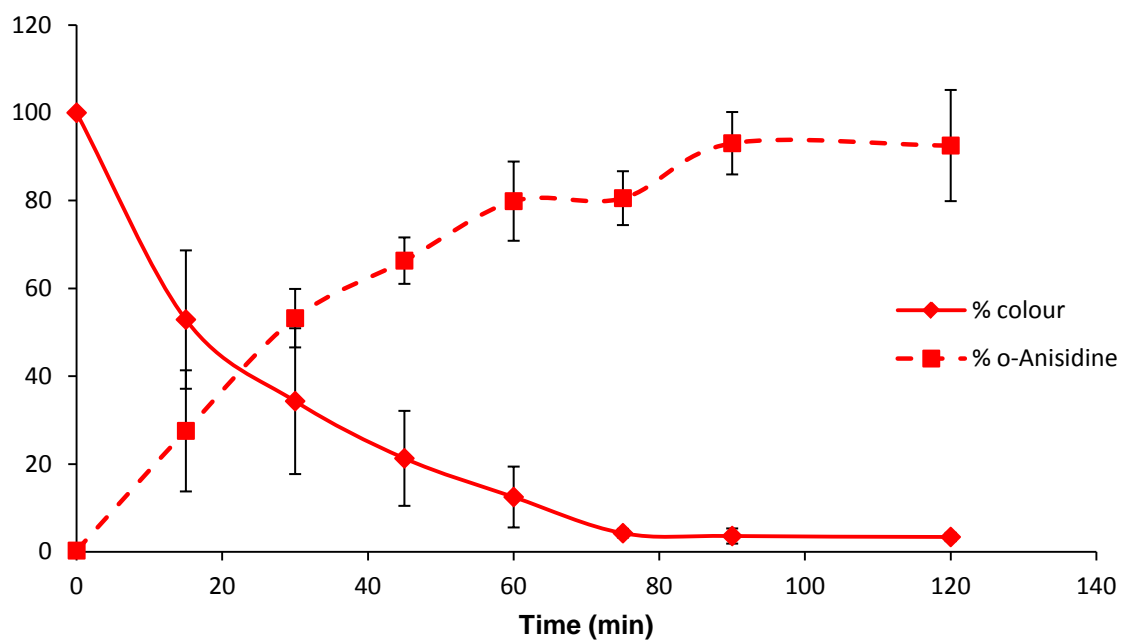


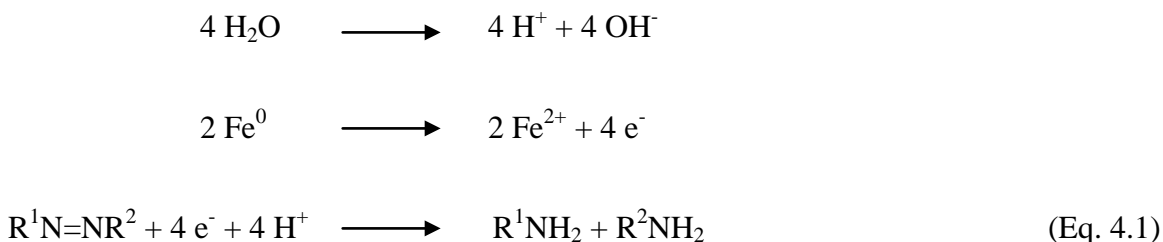
Figure 4.40 Progressive AR4 reduction and *o*-anisidine production with time when treated with 2 g Iron and 1 mM sodium sulfite.

4.5 SBP Treatment of Aniline Recovered from Zero-Valent Iron Reduced Azo-Dye

Iron pre-treatment was shown to be effective in decolourizing both AR4 and COG; however, this method produces anilines which would require treatment before release into the environment. In this section, the aniline and *o*-anisidine, products of iron pretreatment of azo-dyes, were treated by SBP and the treatment optimum were compared to the optimum values for the treatment of authentic anilines.

4.5.1 pH Effect

The anilines recovered solution from the reduction of azo-dyes also contained unreacted sodium sulfite and Fe^{2+} as per Equation 4.1 (Beyedilli et al., 1998). Thus, it was assumed that these substances might affect the parameters of enzymatic treatment by increasing H_2O_2 demand (Mantha, 2001).



Mantha (2001) aerated the solutions after Fe^0 reduction of azo-dyes in order to reduce the effect of Fe^{2+} and sodium sulfite present. In all the experiments in this section the solutions were aerated for 30-50 min, the time it took to run the TNBS test.

COG and AR4 were pretreated with iron as per Section 4.4 and the recovered anilines were analyzed by TNBS test. Since the concentration of azo dyes, pretreated with iron, was 1 mM, and the recovered anilines were less than 1 mM, it was decided to start with

0.5 mM recovered anilines and a pH range of 4.0 to 8.0. Batch reactors had 0.15 U/mL SBP and 1.5 mM H₂O₂ for recovered aniline from COG and 0.009 U/mL SBP and 1.25 mM H₂O₂ for recovered *o*-anisidine from AR4 and were run for 3 hours. The results are shown in Figures 4.41 and 4.42.

Recovered aniline had an optimal pH of 5.0 the same optimal as that of authentic aniline. However, recovered *o*-anisidine showed an optimal pH of 8.0 unlike authentic *o*-anisidine which had an optimal pH of 5.0. The sample with recovered aniline from COG had increased in colour after enzymatic treatment. There was 13% increase in colour after enzymatic treatment at pH 5.0 than after Fe⁰ reduction of COG. While the recovered *o*-anisidine from AR4 had more colour removal after enzymatic treatment with 10% less colour at pH 8.0 than after Fe⁰ reduction. This higher colour removal of AR4 was partly due to the testing at a pH range of 8.0, which was shown in Section 4.4 to be the optimal pH for enzymatic treatment of AR4 without pretreatment. Looking at these results it can be deduced that pretreatment of AR4 with iron gives a better result than direct enzymatic treatment of AR4.

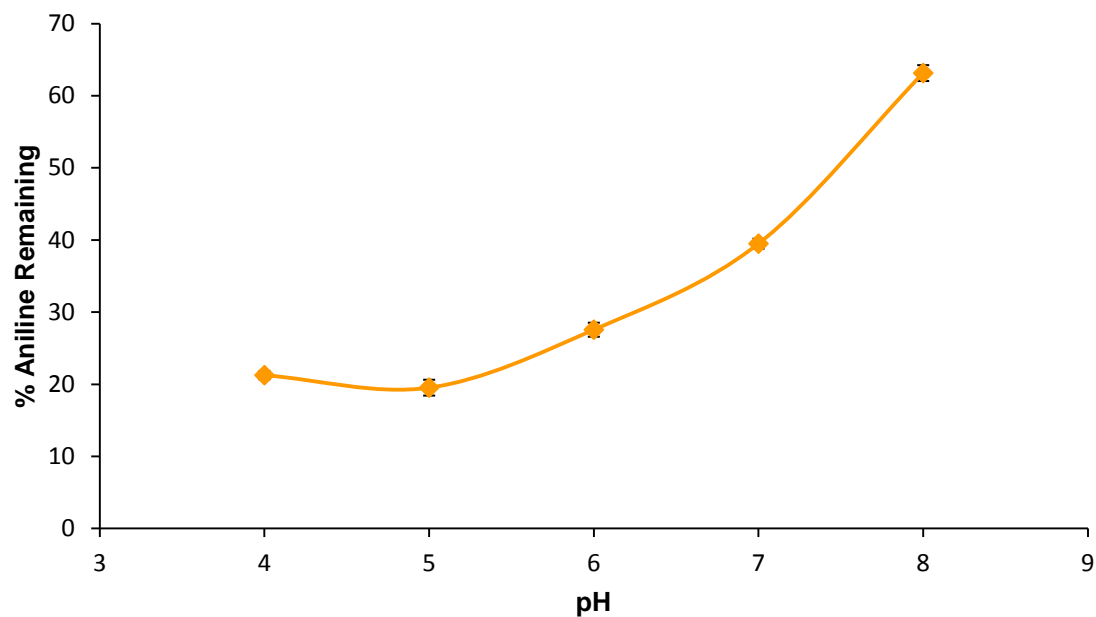


Figure 4.41 pH optimization of 0.5 mM recovered aniline with 0.15 U/mL SBP, 1.5 mM H_2O_2 and reaction time = 3 hr.

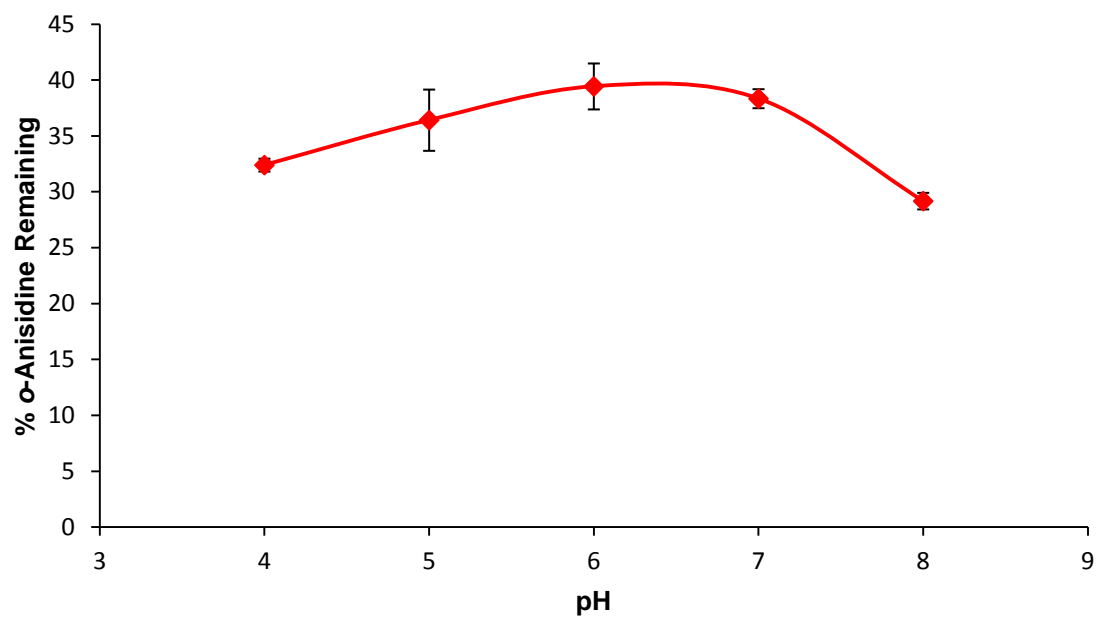


Figure 4.42 pH optimization of 0.5 mM recovered *o*-anisidine with 0.009 U/mL SBP, 1.25 mM H_2O_2 and reaction time = 3 hr.

4.5.2 SBP Dose Optimization

Batch reactor studies were performed to determine the optimal SBP concentration for the removal of 95% of 0.5 mM recovered anilines. The experiments were run for 3 hours at pH 5.0, 1.5 mM H₂O₂ and a SBP range of 0 - 0.4 U/mL SBP for recovered aniline. For recovered *o*-anisidine, the batches were run for 3 hours at pH 8.0 with 1.25 mM H₂O₂ and SBP range of 0 - 0.045 U/mL SBP. Figures 4.43 and 4.44 show the results.

Optimal SBP dose for 94% removal of 0.5 mM recovered aniline was 0.3 U/mL and there was little if any improvement with the addition of more SBP. In the case of 0.5 mM recovered *o*-anisidine, the optimal dose was found to be at 0.035 U/mL with approximately 96% removal and an increase in SBP dose of 0.01 U/mL increased removal to 98%. The increase in *o*-anisidine removal with an increase in SBP dose, which is not seen with aniline, could be related to the 1/10th enzyme concentration needed for the treatment of *o*-anisidine. The catalase activity of the SBP enzyme, discussed by Biswas (1999), could be the factor that would require a higher H₂O₂ uptake with the higher concentration of SBP. This was also reported by Mantha (2001).

For authentic aniline in Section 4.1, optimal dose of 0.6 U/mL SBP was required for the removal of 1 mM aniline. Whereas, recovered *o*-anisidine required 6 times more SBP per mM of *o*-anisidine (0.035 U/mL for 0.5 mM) to achieve 95% removal than authentic *o*-anisidine, which required 0.0116 U/mL SBP for the treatment of 1 mM. The need for the extra SBP could be due to the complex nature of the solution in which further colour reduction was observed, indicating that some fraction of the SBP was treating colour and thus extra SBP was required.

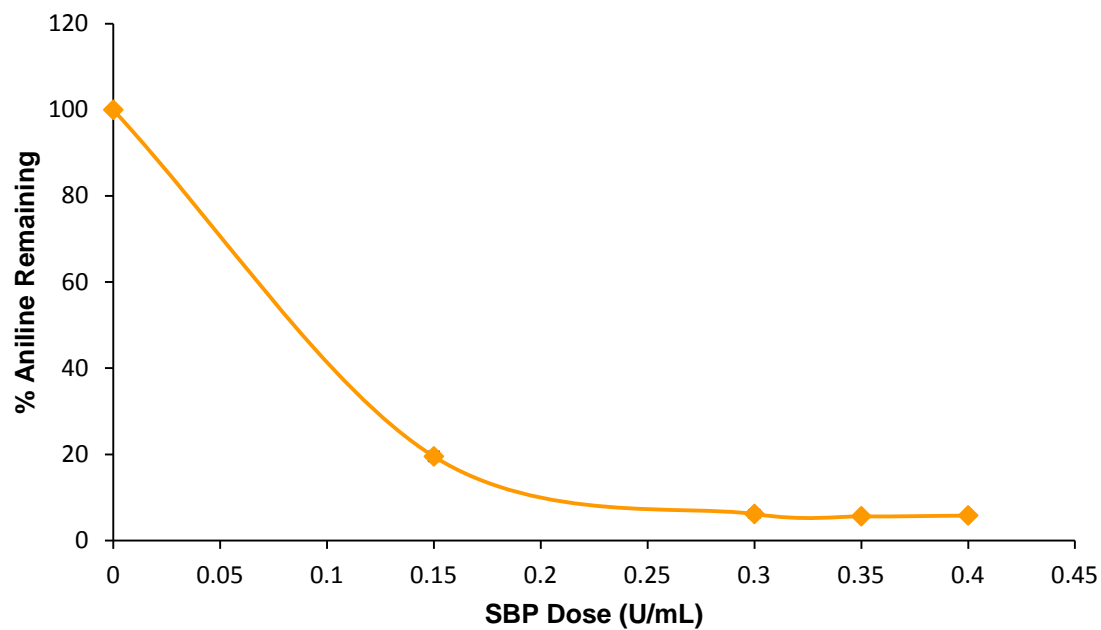


Figure 4.43 SBP dose optimization for treatment of 0.5 mM recovered aniline at pH 5.0 with 1.5 mM H_2O_2 and reaction time = 3 hr.

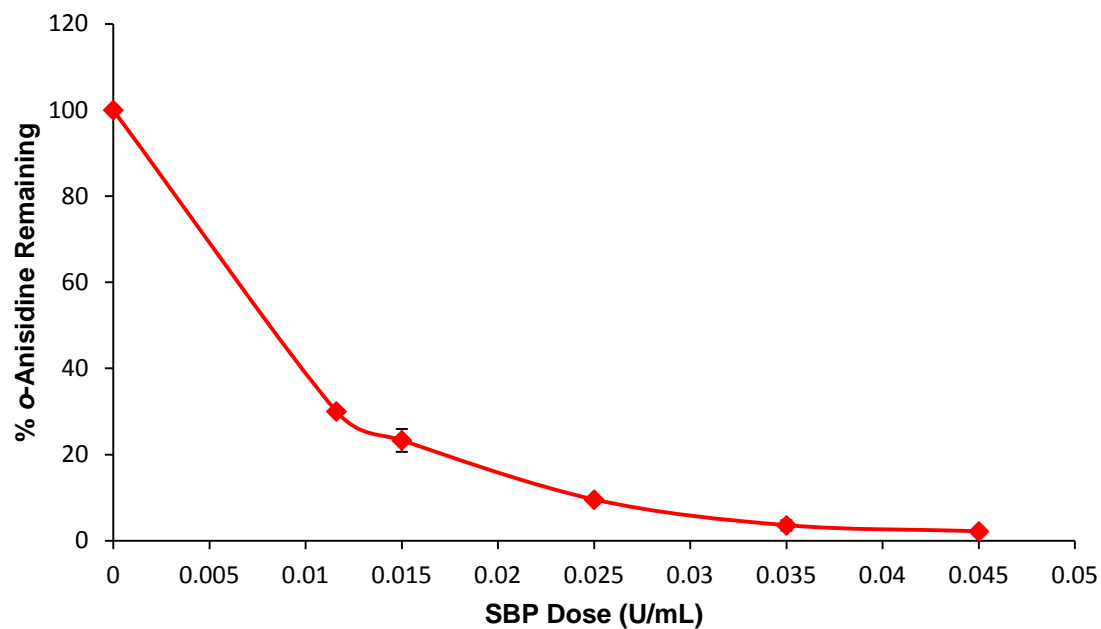


Figure 4.44 SBP dose optimization for treatment of 0.5 mM recovered *o*-anisidine at pH 8.0 with 1.25 mM H_2O_2 and reaction time = 3 hr.

4.5.3 H₂O₂ to Substrate Ratio Optimization

In Section 4.1.3 the optimal H₂O₂ to substrate ratio was 1.5 for authentic aniline and 1.25 for *o*-anisidine. To determine the optimal ratios for 0.5 mM recovered aniline and *o*-anisidine, batches were set up at optimal pH and SBP dose as per Section 4.5.1 and 4.5.2 and a range of H₂O₂ concentration from 0.5 - 2 mM was used. The results are plotted in Figures 4.45 and 4.46.

As was observed with authentic aniline and *o*-anisidine in Section 4.1.3, SBP was able to treat both anilines in the full H₂O₂ range studied. However, in both cases the H₂O₂ to substrate ratio required to achieve optimal results was twice that found for authentic anilines. The results show an optimal of 3:1 for recovered aniline and 2-2.5:1 for recovered *o*-anisidine. This increase in H₂O₂ demand could be due to the complex nature of the iron-reduced azo-dye solution. During zero-valent iron reduction of the dyes, azo-bonds were split resulting in two compounds, an aniline or *o*-anisidine and an amino-naphthol sulfonic acid compound, as well some of the original colour remained. All these compounds are substrates for the enzyme, and as a result would require more H₂O₂ to treat. Another contributor for the increase in H₂O₂ demand could be a result of sodium sulfite and Fe²⁺ remaining from the Fe⁰ reduction process. All these factors might have led to the doubling of H₂O₂ demand.

As with authentic aniline and *o*-anisidine, as H₂O₂ to substrate ratio increased past the optimal values the efficiency of removal decreased. This gives further proof for SBP inactivation by H₂O₂. Overall, the enzymatic treatment of recovered aniline at optimal

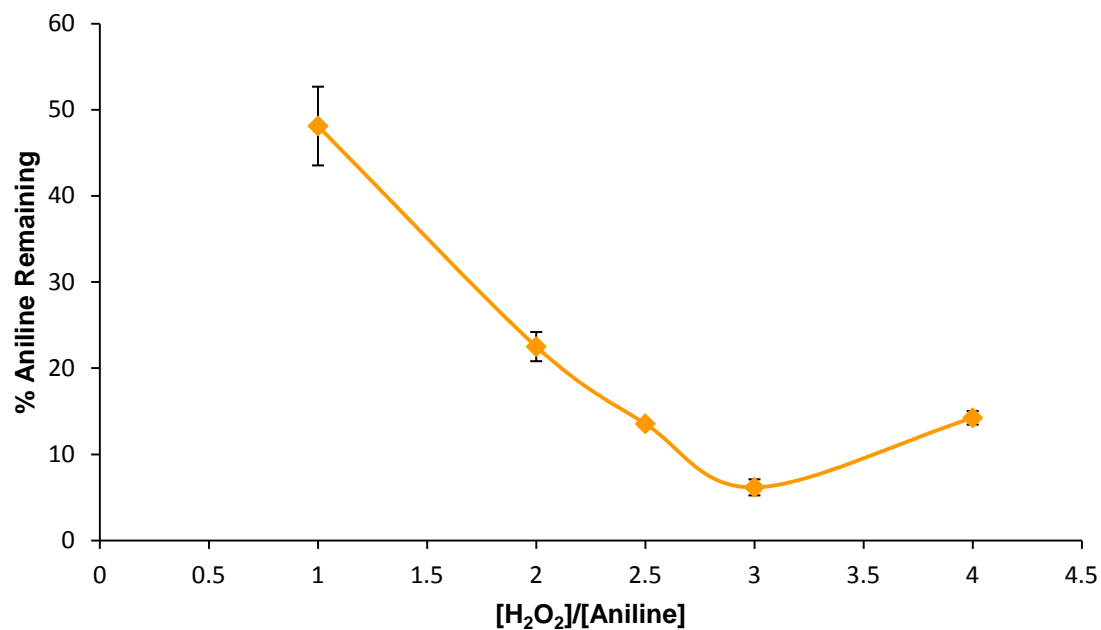


Figure 4.45 Optimization of hydrogen peroxide to substrate ratio for removal of 0.5 mM recovered aniline at pH 5.0 with 0.3 U/mL SBP and reaction time = 3 hr.

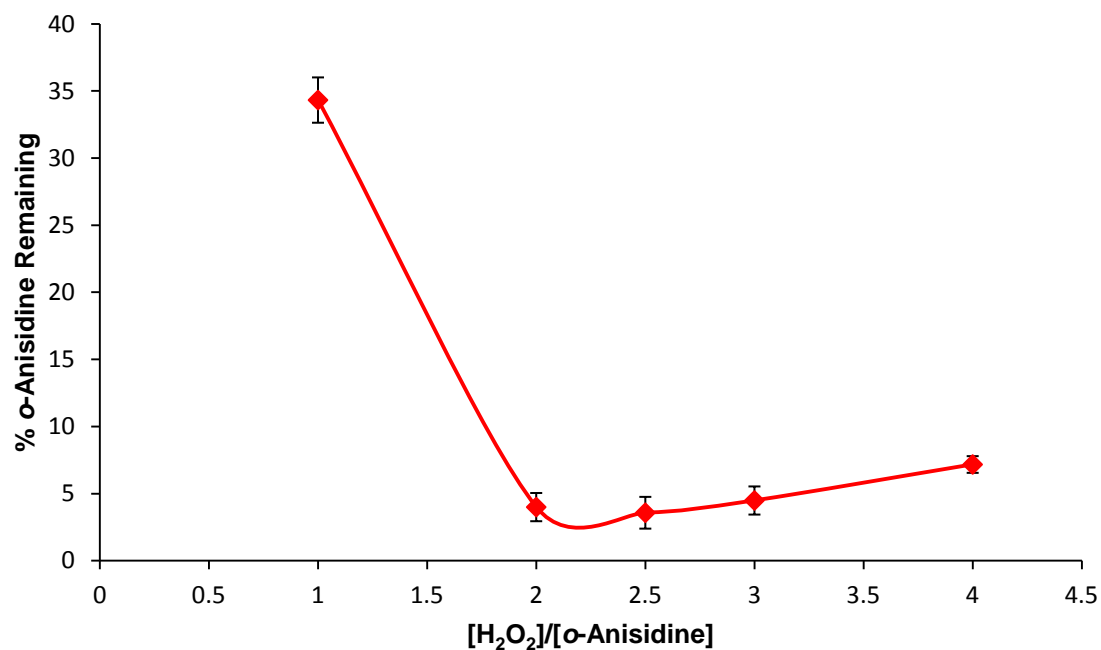


Figure 4.46 Optimization of hydrogen peroxide to substrate ratio for removal of 0.5 mM recovered *o*-anisidine at pH 8.0 with 0.035 U/mL SBP and reaction time = 3 hr.

conditions resulted in an increase in colour, while the enzymatic treatment of recovered *o*-anisidine resulted in further decolourization.

4.5.4 SDS Effect

The addition of SDS was shown in Section 4.2 to give the best results, as compared to the other additives studied, for enzymatic treatment of aniline and *o*-anisidine. Thus, the effect of SDS on SBP treatment of recovered aniline and *o*-anisidine was studied in this section. After zero-valent iron pretreatment of COG and AR4, the recovered anilines were analyzed by TNBS test and 0.5 mM was added to 20 mL batch reactors. Reactors were run for 3 hours at pH 5.0 with 0.5 U/mL SBP and 1.5 mM H₂O₂ for aniline or at pH 8.0 with 0.015 U/mL SBP and 1.25 mM H₂O₂ for *o*-anisidine with a range of SDS from 0 - 300 mg/L. The results for recovered aniline showed no SDS effect. Therefore further experiments were run by varying the pH and maintaining SDS concentration at 100 mg/L which again showed no SDS effect. The results for the effect of SDS on recovered *o*-anisidine are shown in Figure 4.47.

The best effect of SDS on the removal of 0.5 mM recovered *o*-anisidine was seen at 50 mg/L and any addition of SDS above that did not improve treatment. Thus, it was determined that, for this study, the optimal SDS concentration would be 50 mg/L for 0.5 mM recovered *o*-anisidine, which is similar to authentic *o*-anisidine which showed optimal SDS effect of 100 mg/L for 1 mM *o*-anisidine.

Optimal enzymatic treatment of *o*-anisidine in the presence of SDS for authentic *o*-anisidine was seen at a slightly different optimal pH than with no SDS addition. Therefore, in Figure 4.48 the effect of pH on recovered *o*-anisidine enzymatic treatment

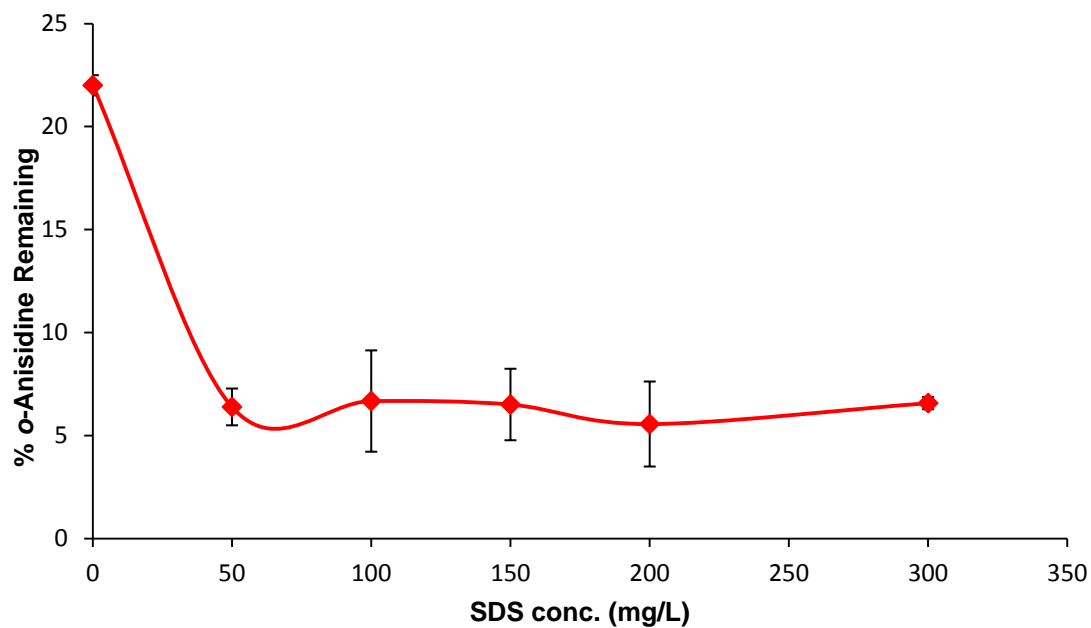


Figure 4.47 SDS effect on 0.5 mM recovered *o*-anisidine treatment at pH 8.0 and 0.015 U/mL SBP with 1.25 mM H₂O₂ and reaction time = 3 hr.

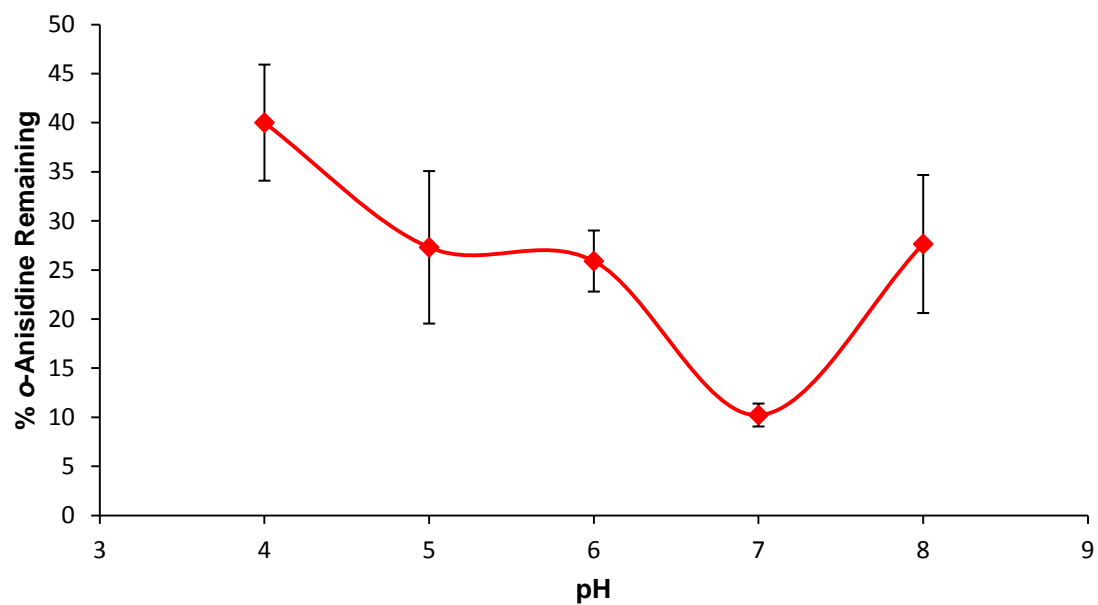


Figure 4.48 pH optimization of 0.5 mM recovered *o*-anisidine removal in presence of 50 mg/L SDS and 0.007 U/mL SBP with 1.25 mM H₂O₂ and reaction time = 3 hr.

in the presence of SDS was examined. The batches were set up with range of pH of 4.0 to 8.0, with 0.007 U/mL SBP, 1.25 mM H₂O₂ in the presence of 50 mg/L SDS and were run for 3 hours.

As with authentic *o*-anisidine, the optimal pH in the presence of SDS was different than without its addition. In the presence of 50 mg/L SDS the optimal pH was seen at pH 7.0. The addition of SDS shows more pH-dependent enzymatic treatment; 30% more removal at optimum pH than at worst-case pH with no addition and 10% more removal than worst-case pH with addition. This behaviour was also observed by Al Ansari et al. (2010a) in the SBP treatment of phenol in the presence of SDS. They speculated that the presence of SDS could slightly change the structural conformation of SBP leading to a change in the optimum pH.

To determine the SDS effect on H₂O₂ to substrate ratio, batch reactors were run for 3 hours with 0.5 mM recovered *o*-anisidine, 0.022 U/mL SBP, pH 7.0, and a H₂O₂ concentration range of 0.5 to 2 mM in the presence of 50 mg/L SDS. The results, in Figure 4.49 show that the optimal H₂O₂:substrate was 2.5:1. This is similar to that obtained in the absence of SDS; however, at 0.022 U/mL SBP there was approximately 100% *o*-anisidine removal. Thus experiments were set up to determine SBP dose for the 95% removal. As with earlier experiments to optimize SBP dose, all parameters were held at optimal concentrations and SBP dose was varied, in this case 0.005 - 0.022 U/mL. The results shown in Figure 4.50 indicate that 0.011 U/mL SBP is needed for 95% removal of recovered *o*-anisidine which is 1/3 of enzyme required without SDS. Thus, the addition of SDS in SBP-catalyzed treatment is seen to be very effective in reducing

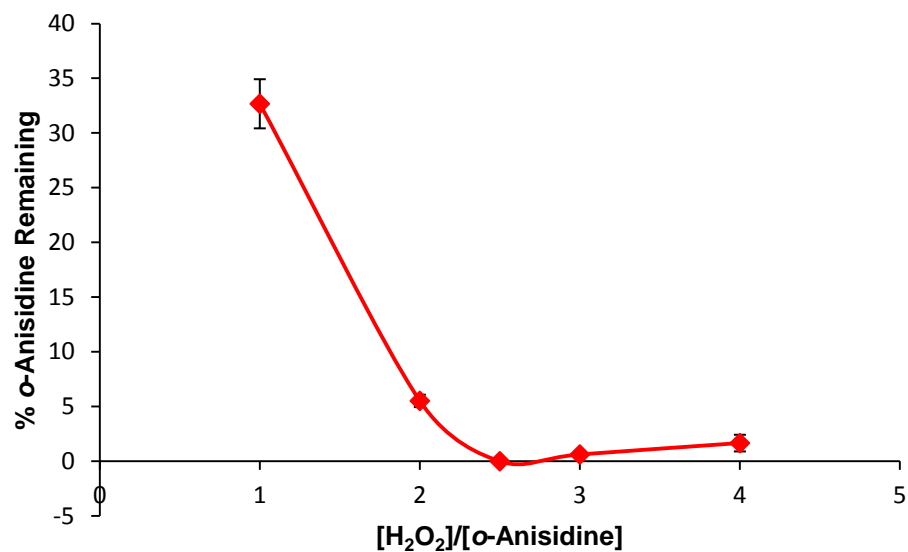


Figure 4.49 Hydrogen peroxide to substrate ratio optimization of 0.5 mM recovered *o*-anisidine at pH 7.0 in presence of 50 mg/L SDS, 0.022 U/mL SBP and reaction time = 3 hr.

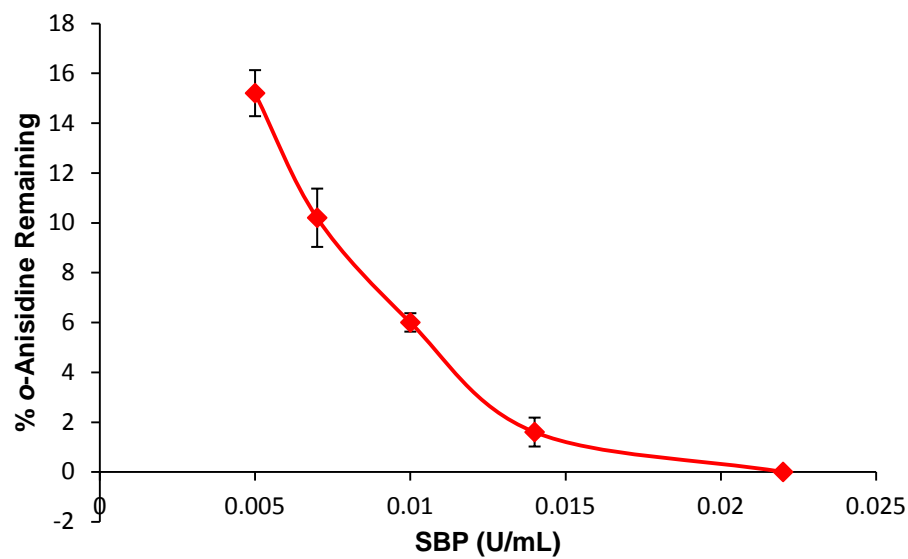


Figure 4.50 SBP optimization of 0.5 mM recovered *o*-anisidine at pH 7.0 in presence of 50 mg/L SDS, with 1.25 mM H₂O₂ and reaction time = 3 hr.

reducing enzyme concentration. The treatment of authentic aniline with SBP was enhanced in the presence of SDS, unlike recovered aniline. This could be due to the complex nature of the solution. A summary of the results are given in Table 4.11.

Table 4.11 Summary of optimal parameters for the treatment of recovered anilines.

Substrate	Additive	pH	[SBP] U/mL	[H ₂ O ₂]:[substrate] (mM:mM)
Recovered Aniline	None	5.0	0.3	3:1
	SDS	No effect	No effect	No effect
Recovered <i>o</i> -Anisidine	None	8.0	0.035	2-2.5:1
	SDS	7.0	0.011	2.5:1

* Starting with 0.5 mM recovered anilines and time = 3 hours

Overall, zero-valent iron pre-treatment of COG and AR4 was shown to be effective in reducing both colour and recovered anilines. The results of colour reduction can be seen in the UV-VIS spectra of normalized concentrations in Figure 4.51 and 4.52 at $\lambda_{\text{max}} = 508$ nm for AR4 and at $\lambda_{\text{max}} = 482$ nm for COG. COG was diluted to 25 μM and AR4 was diluted to 50 μM for all curves. It can be seen that this two-step process was more effective in the treatment of AR4 than treatment of COG. SBP treatment of recovered anilines from Fe^0 reduced COG, increased colour intensity, whereas, SBP treatment of recovered *o*-anisidine from Fe^0 reduced AR4, decreased colour intensity.

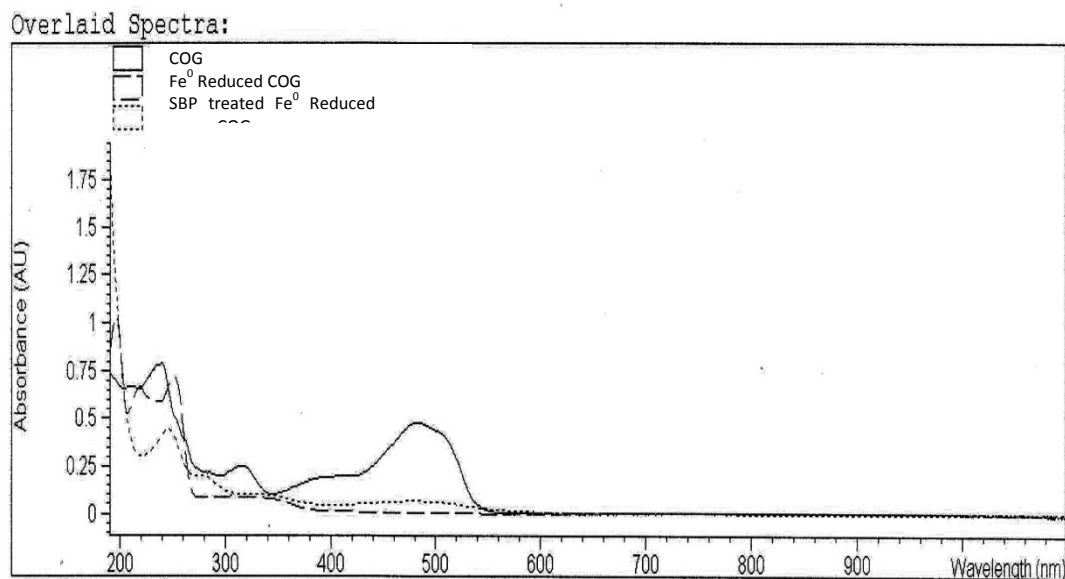


Figure 4.51 Zero-valent reduction COG decolourization followed by SBP catalyzed aniline treatment.

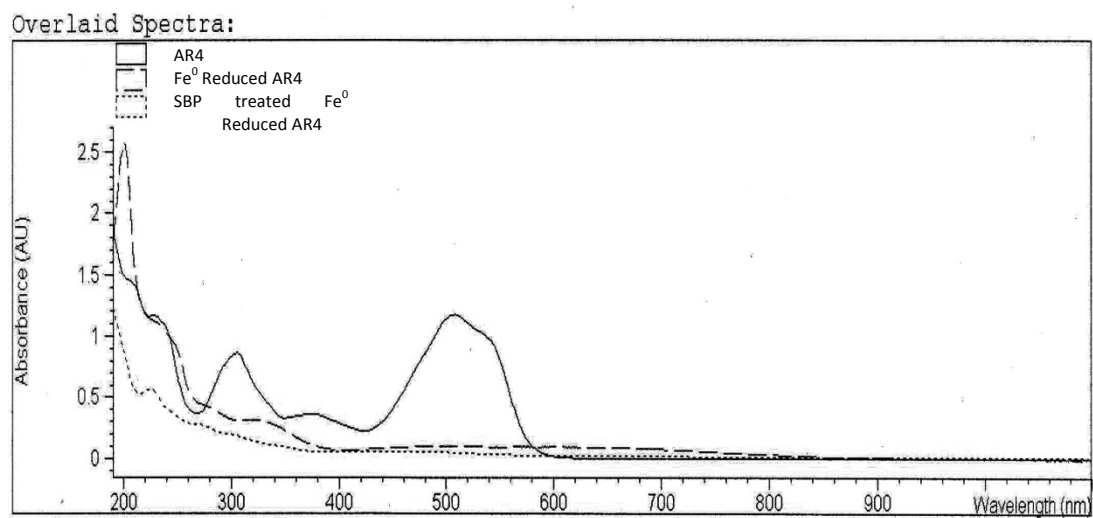


Figure 4.52 Zero-valent iron reduction AR4 decolourization followed by SBP catalyzed *o*-anisidine treatment

CHAPTER 5

Summary & Conclusions

The results of this study demonstrate the ability of SBP to remove azo-dyes and their reduction products during wastewater treatment. Aniline and *o*-anisidine were optimized for 95% removal through SBP-catalyzed polymerization and additives were able to decrease the amount of enzyme needed to achieve these optima. Both dyes, COG and AR4, were successfully decolourized by direct treatment with SBP. Subsequently, zero-valent iron reduction of both dyes was carried out; it was possible to decolourize both dyes and their reduction product, anilines, were successfully treated with SBP.

The SBP treatment of authentic aniline and *o*-anisidine was optimized for pH, H₂O₂:substrate ratio, and enzyme dose. It was shown that SBP was active over a broad range of pH (3.5-7.5) with an optimal range of 4.5 to 5.5 and optimal removal was achieved at pH 5.0 for both substrates. However, the treatment of *o*-anisidine was more sensitive to pH changes than aniline. The SBP concentration required to achieve 95% removal of aniline was 0.6 U/mL, while for *o*-anisidine removal the concentration was approximately 50 times lower at 0.0116 U/mL. The H₂O₂ to substrate molar ratio required for aniline was found to be 1.5, while that for *o*-anisidine was 1.25. It was also found that the amount of H₂O₂ required increased with increase in enzyme dose.

Additives were shown to be effective in reducing the SBP dose required in SBP-catalyzed polymerization of aniline and *o*-anisidine. Among the different additives studied, PEG was the only one that had no effect. SDS addition decreased the required SBP dose by approximately 2-fold and had the additional benefit of improved precipitate formation, settling and colour removal, while slowing initial reaction rate and increasing SBP catalytic life. However, the presence of SDS also affected the optimal pH range, with a change in pH having more drastic impact on removal than without SDS. From TOC studies, it was concluded that the addition of SDS improved precipitation and settling more products than without SDS addition.

Azo-dyes were decolourized successfully with SBP treatment. SBP decolourized both azo-dyes well at a pH range of 3.5 - 4.0 and 8.0 - 8.5. Optimization was done at pH 8.0, with the addition of 0.75 U/mL SBP and 3.5 mM H₂O₂ for 1 mM dye when 95% colour removal for COG and 85% colour removal for AR4 were achieved. An increase in SBP or H₂O₂ concentration did not improve treatment efficiency.

Zero-valent iron reduction of both azo-dyes was demonstrated to reduce over 95% of the colour. For 40 mL of 1.0 mM COG, optimal conditions were the addition of 1 g iron, 1 mM sodium sulfite and contact time of 75 min, while optimal conditions for 40 mL at 1.0 mM AR4 included the addition of 2 g iron, 1 mM sodium sulfite and run for 90 min. COG showed lower product recovery than AR4, which indicated more product adsorbed on iron in COG reduction than in AR4 reduction.

Further treatment of the anilines recovered from zero-valent iron reduction of COG and AR4 was successful through SBP-catalyzed polymerization. The products of reduction

investigated were aniline for COG and *o*-anisidine for AR4. The other products that were not part of this investigation were amino naphthol sulfonic acid compounds. There was a preliminary investigation as part of an undergraduate study that showed potential for these compounds to be substrates of SBP, however these would be subject to further study. At an optimal pH of 5.0, recovered aniline required the same amount of SBP as authentic aniline; however, the amount of H₂O₂ required was doubled, which indicates parallel treatment of the other products of reduction. SBP treatment of recovered *o*-anisidine, did not behave like authentic *o*-anisidine. At optimal pH of 8.0, six times the amount of SBP and double the amount of H₂O₂ were required to achieve 95% removal. The low purity, 45%, of AR4 was attributed to this change, as well as parallel treatment of other products of reduction. The presence of SDS did not improve the enzymatic treatment of recovered aniline; however, 3-fold lower SBP concentration was required for the treatment of *o*-anisidine at an optimal pH of 7.0.

It can be concluded from these results that both direct treatment of azo-dyes with SBP or pretreatment with iron followed by SBP-catalyzed polymerization provide a successful means to decolourize and treat these azo-dyes. Direct treatment of 1 mM COG at pH 8.0 with SBP is the preferred method, due to its ability to achieve 95% colour reduction at an SBP concentration of 0.75 U/mL and H₂O₂ concentration of 3.5 mM. The pretreatment of 1 mM COG with iron followed by SBP treatment of 0.5 mM recovered aniline required 0.6 U/mL SBP to achieve 95% removal but it increased the residual colour. Direct treatment of AR4 with SBP was unable to achieve 95% removal, even at high SBP doses. Thus, 1 mM AR4 can be treated best by zero-valent iron reduction followed by SBP-catalyzed polymerization of the recovered *o*-anisidine in the presence of SDS at pH

7.0. In this method only 0.011 U/mL SBP with 1.25 mM H₂O₂ was required to achieve >95% removal of both 0.5 mM *o*-anisidine and colour.

CHAPTER 6

Recommendations

The results of this study validate the use of SBP, either directly or after pre-treatment with zero-valent iron, to catalyze removal of azo-dyes and their reduction products in wastewater treatment. However, before this treatment can be implemented as an alternative to existing wastewater treatment methods, further studies have to be done.

1. The effect of additives, was only studied on authentic anilines and those recovered after zero-valent iron reduction. Thus the effect in direct enzymatic treatment of azo-dyes could potentially reduce SBP dose and enhance decolourization.
2. Studies on toxicity of both soluble and insoluble products should be conducted along with impact of these products on the environment as well as downstream treatment.
3. The mechanism of additive effect on enhancing SBP-catalyzed treatment should be further investigated. Such as, quantifying the quality of precipitate formed through time for settling, light scattering and UV-Vis spectra. Studies on enzyme activity in the presence of additives at various pH values could be of benefit.

4. SBP-catalyzed treatment of composite water containing a mixture of dyes or a real wastewater sample from industry should be investigated to determine the level of conversion of each substrate and the existence of any matrix effects.
5. The other reduction products of zero-valent iron treatment of azo-dyes, the naphthyl compounds, should be further identified, quantified and the extent of their enzymatic treatment should be determined.
6. Zero-valent iron column reduction studies on azo-dye wastewater should be carried out to aid in the design of a system that could be applied in industry.
7. Desorption of reduction products from iron, as well as regeneration of iron to reduce cost, improve process efficiency and column life should be investigated.
8. Cost analysis of the process is essential in order to promote application in industry.

References

- ACGIH (1991) Documentation of the threshold limit values and biological exposure indices, sixth edition. American Conference of Governmental Industrial Hygienists, Inc., Cincinnati, OH, pp. 256-257.
- Agrawal, A. and Tratnyek, P. G. (1996) Reduction of nitro aromatic compounds by zero-valent iron metal. *Environmental Science and Technology* 30(1):153-160.
- Ahn, D, Chang, W., and Yoon, T. (1999) Dyestuff wastewater treatment using chemical oxidation, physical adsorption and fixed bed biofilm process. *Process Biochemistry* 34(5): 429-439.
- Al-Ansari, M. M. (2008) Removal of various compounds from synthetic and refinery wastewater using soybean peroxidase. Master of Science Thesis. University of Windsor, Windsor, Ontario, Canada.
- Al-Ansari, M. M., Saha, B., Mazloun, S., Taylor, K., Bewtra, J., Biswas, N. (2011) Soybean peroxidase applications in wastewater treatment. In: *Soybean Cultivation, Uses & Nutrition*. Maxwell, J. F. (ED). USA, Nova Science Publishers, Chapter 6.
- Al-Ansari, M. M., Modaressi, K., Taylor, K. E., Bewtra, J. K. and Biswas, N. (2010a) Soybean peroxidase-catalyzed oxidative polymerization of phenols in coal-tar wastewater: comparison of additives. *Environmental Engineering Science* 27(11): 967-975.
- Al-Ansari, M.M., Steevensz, A., Tarylor., K. E., Bewtra, K. E. and Biswas, N. (2010b) Soybean peroxidase-catalyzed removal of an aromatic thiol, 2-mercaptobenzothiazole, from water. *Water Environment Research* 82(11): 2285-2289.
- Al-Ansari, M. M., Steevensz, A., Al-Aasm, N., Taylor, K. E., Bewtra, J. K., and Biswas, N. (2009) Soybean peroxidase-catalyzed removal of phenylenediamines and benzenediols from water. *Enzyme and Microbial Technology* 45(4): 253-260.
- Arano, M. B., Acosta, M., del Rio, J. A., Varon, R. and Garcia-Canovas, F. (1990) A kinetic study on the suicide inactivation of peroxidase by hydrogen peroxide. *Biochimica et Biophysica Acta (BBA)* 1041(1): 43-47.
- Arslan-Alaton, I. (2003) The effect of pre-ozonation on the biocompatibility of reactive dye hydrolysates. *Chemosphere* 51(9): 825-833.
- Atiken, M. D. (1993) Waste treatment applications of enzymes: opportunities and obstacles. *The Chemical Engineering Journal* 52(2): B49-58.
- ATSDR (Agency for Toxic Substances & Disease Registry) (2002) Aniline: Division of Toxicology ToxFAQsTM. U.S. Department of Health and Human Services, Public Health Service. <http://www.atsdr.cdc.gov/toxfaqs/tfacts171.pdf>. Last accessed March 3, 2014.

Baek, H. K. and Van Wart, H. E. Elementary steps in the formation of horseradish peroxidase Compound I: direct observation of Compound 0, a new intermediate with a hyperporphyrin spectrum. *Biochemistry* 28(14): 5714-5719.

Bafana, A., Devi, S. S. and Chakrabarti, T. (2011) Azo dyes: past, present and the future. *Environmental Reviews* 19(1): 350-370.

Baird, R., Carmona, L., and Jenkins, R. L. (1977) Behavior of benzidine and other aromatic amines in aerobic wastewater treatment. *Journal (Water Pollution Control Federation)* 49(7): 1609-1615.

Banat, I. M., Nigam, P., Singh, D., and Marchant, R. (1996) Microbial decolorization of textile-dye-containing effluents: a review. *Bioresource Technology* 58(3): 217-227.

Bassi, A., Geng, Z. and Gijzen, M. (2004) Enzymatic removal of phenol and chlorophenols using soybean seed hulls. *Engineering in Life Science* 4(2): 125-130.

Beard, R. R. and Noe, J. T. (1981) Aromatic nitro and amino compounds. In: *Patty's Industrial Hygiene and Toxicology*, 3rd Edit., Vol. 2A. Clayton, G. D., and Clayton, F. E.(Eds). New York, John Wiley & Sons, pp. 2413-2446.

Bhunia, A., Durani, S., and Wangikar, P. P. (2001) Horseradish peroxidase catalyzed degradation of industrially important dyes. *Biotechnology and Bioengineering* 72(5): 562-567.

Biswas, K. (1999) Removal of Cresols from Synthetic wastewater using crude soybean peroxidase. M.A.Sc. Thesis. University of Windsor, Windsor, Ontario, Canada.

Biswas, M. M. (2004) Removal of reactive azo dyes from water by Fe^0 reduction followed by peroxidase catalysed polymerisation. M.A.Sc. Thesis. University of Windsor, Windsor, Ontario, Canada.

Biswas, M. M., Biswas, N., Bewtra, J. K. and Taylor, K. (2004) Removal of reactive azo dyes from water by Fe^0 reduction followed by peroxidase-catalysed polymerisation. *Water Environment Federation 10th Annual Industrial Wastes Technical and Regulatory Conference*.

Biswas, M. M., Taylor, K. E., Bewtra, J. K., and Biswas, N. (2007) Enzymatic treatment of sulfonated aromatic amines generated from reductive degradation of reactive azo dyes. *Water Environment Research* 79(4): 351-356.

Bodalo A., Gomez, J. L., Gomez, E., Bastida, J. and Maximo, M. F. (2006) Comparison of commercial peroxidases for removing phenol from water solutions. *Chemosphere* 63(4): 626-632.

Cantrell, K. J., Kaplan, D. I. and Weitsma, T. W. (1995) Zero-valent iron for the in situ remediation of selected metals in groundwater. *Journal of Hazardous Materials* 4(2): 201-212.

Cao, H. (2000) Decolorization of textile dyes by white rot fungi. Ph.D. Dissertation. University of Georgia, Athens, Georgia, USA.

Cao, J., Wei, L., Huang, Q., Wang, L. and Han, S. (1999) Reducing degradation of azo dye by zero-valent iron in aqueous solution. *Chemosphere* 38(3): 565-571.

Caza, N., Bewtra, J. K., Biswas, N. and Taylor, K. E. (1999) Removal of phenolic compounds from synthetic wastewater using soybean peroxidase. *Water Research* 33(13): 3012-3018.

Carliell, C. M., Barclay, S. J., Naldoo, N., Buckley, C. A., Mulholland, D. A. and Senior, E. (1995) Microbial decolourisation of a reactive azo dye under anaerobic conditions. *Water SA* 21(1): 61-69.

Carliell, C. M., Barclay, S. J., and Buckley, C. A. (1996) Treatment of exhausted reactive dye bath effluent using anaerobic digestion: laboratory and full scale trials. *Water SA* 22(3): 225-233.

Casero, I., Sicilia, D., Rubio, S., and Perez-Bendito, D. (1997) Chemical degradation of aromatic amines by fenton's reagent. *Water Research* 31(8): 1985-1995.

Cheng, I. F., Muftikian, R., Fernando, Q. and Korte, N. (1997) Reduction of nitrate to ammonia by zero-valent iron. *Chemosphere* 35(11): 2689-2695.

Choe, S., Lee, S., Chang, Y., Hwang, K. and Khim, J. (2001) Rapid reductive destruction of hazardous organic compounds by nanoscale Fe^0 . *Chemosphere* 42(4): 367-372.

Chudgar, R. J. and Oakes, J. (2001) Dyes, azo. In: *The Kirk-Othmer Encyclopedia of Chemical Technology*, John Wiley and Sons, New York. Published online Nov 14, 2003. <http://onlinelibrary.wiley.com/doi/10.1002/0471238961.01261503082104.a01.pub2/abstract>. Last accessed: March 6, 2014.

Coughlin, M. F., Kinkle, B. K., and Bishop (1999) Degradation of azo dyes containing aminonaphthol by *Sphingomonas* sp strain 1 CX. *Journal of Industrial Microbiology and Biotechnology* 23(4): 341-346.

Dahghani, M. H., Jaafari, J., Alghasi, A., and Porkar, G. (2011) Using medium pressure ultraviolet reactor for removing azo dyes in textile wastewater treatment plant. *World Applied Sciences Journal* 12 (6): 797-802.

Demarche, P., Junghanns, C., Nair, R. R., and Agathos, S. N. (2012) Harnessing the power of enzymes for environmental stewardship. *Biotechnology Advances* 30(5): 933-953.

Dunford, H. B. (1999) Peroxidases & catalases: biochemistry, biophysics and physiology 2nd edition. Hoboken, NJ. John Wiley & Sons. pp 1-56.

Dunford H. B. and Stillman, J. S. (1976) On the function and mechanism of action of peroxidases. *Coordination Chemistry Reviews* 19: 187-251.

Environment Canada (2012) The chemicals management plan substance grouping initiative- aromatic azo- and benzidine-based substances. Environment Canada. http://www.ec.gc.ca/ese-ees/9E759C59-55E4-45F6-893A-F819EA9CB053/Azo_Technical%20Background_EN.pdf. Last accessed: March 6, 2013.

European Commission (2011) Recommendation from the scientific committee on occupational exposure limits for o-anisidine. European Commission Employment, Social Affairs and Inclusion. ec.europa.eu/social/BlobServlet?docId=7422&langId=en. Accessed March 5, 2014.

European Commission (2002) European union risk assessment report: o-anisidine. European Chemicals Bureau Existing Substances 2nd Priority List, Vol. 15. Austria. European Communities.

EU (European Union) (2009) Commission Regulation (EC) No 552/2009 of 22 June 2009 amending Regulation (EC) No 1907/2006 of the European Parliament and of the Council on the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH) as regards Annex XVII. Official Journal of the European Union L 164:7–31.

EU (European Union) (2006) Regulation (EC) No 1907/2006 of the European Parliament and of the Council of 18 December 2006 concerning the Registration, Evaluation, Authorisation and Restriction of Chemicals (REACH), establishing a European Chemical Agency, amending Directive 1999/45/EC and repealing Council Regulation (EEC) No 793/93 and Commission Regulation (EC) No 1488/94 as well as Council Directive 76/769/EEC and Commission Directives 91/155/EEC, 93/67/EEC, 93/105/EC and 2000/21/EC. Official Journal of the European Union L 396:1–849.

Feng, W., Taylor, K. E., Biswas, N. and Bewtra, J. K. (2013) Soybean peroxidase trapped in product precipitate during phenol polymerization retains activity and may be recycled. *Journal of Chemical Technology and Biotechnology* 88(8):1429-1435.

Flock, C., Bassi, A., and Gizen, M. (1999) Removal of aqueous phenol and 2-chlorophenol with purified soybean peroxidase and raw soybean hulls. *Journal of Chemical Technology Biotechnology* 74(4): 303-309.

Flores-Loyola, E., Cruz-Silva, R., Romero_Garcia, J., Angulo-Sanchez, J. L., Castillon, F. F. and Farias, M. H. (2007) Enzymatic polymerization of aniline in the presence of different inorganic substrates. *Materials Chemistry and Physics* 105(1): 136-141.

Gillham, R. W. and O'Hannesin, S. F. (1992) Metal-catalysed abiotic degradation of halogenated organic compounds. IAH Conference "Modern trends in hydrogeology." Hamilton, Ontario, May 10-13. pp. 94-103.

Gillham, R. W. and O'Hannesin, S. F. (1994) Enhanced degradation of halogenated aliphatics by zero-valent iron. *Ground Water* 32(6): 958-967.

Gizen, M., van Huystee, R. and Buzzel, R. I. (1993) Soybean seed coat peroxidase: a comparison of high-activity and low-activity genotypes. *Plant Physiology* 103(4): 1061-1066.

Gomez, J. L., Bodalo, A., Gomez, E., Bastida, J., Hidalgo, A. M. and Gomez, M. (2006) Immobilization of peroxidases on glass beads: An improved alternative for phenol removal. *Enzyme and Microbial Technology* 39(5): 1016-1022.

Government of Canada (2010) Canadian environmental protection act: Follow-up assessment report: Aniline. Environment Canada. http://www.ec.gc.ca/lcpe-cepa/7B18E56F-6BEE-4551-AE33-D7B02F45A6D9/Aniline_Follow_Up_EN.pdf. Accessed April 13, 2014.

Government of Canada (2002) Canadian environmental protection act, 1999: Follow-up report on a PSL1 substance for which data were insufficient to conclude whether the substance was 'toxic' to human health: Aniline. http://www.ec.gc.ca/ese-ees/937FF8BF-BD5D-4864-B62E-50436CAD9484/Aniline_EN.pdf. Accessed March 3, 2014.

Government of Canada (1994) Canadian environmental protection act: Priority substances list assessment report: Aniline. Cat. no. En40-215/35E. Beauregard Printers Limited. http://www.hc-sc.gc.ca/ewh-semt/alt_formats/hecs-sesc/pdf/pubs/contaminants/psl1-lsp1/aniline/aniline-eng.pdf. Accessed March 3, 2014.

Hartman, G. L., West, E. D. and Herman, T. K. (2011) Crops that feed the world 2. Soybean- worldwide production, use, and constraints caused by pathogens and pests. *Food Security* 3(1): 5-17.

Hailu, G., Weersink, A., and Cahlik, F. (2010) Examining the prospects for commercialization of soybean peroxidase. *The Journal of Agrobiotechnology Management & Economics* 13(3): 263-273.

Huang, C. R., Lin, Y. K., and Shu, H. Y. (1994) Wastewater decolorization and TOC-reduction by sequential treatment. *American Dyestuff Reporter* 83(10): 15-18.

Husain, Q., Husain, M., and Kulshrestha, Y. (2009) Remediation and treatment of organopollutants mediated by peroxidases: a review. *Critical Reviews in Biotechnology* 29(2): 94-119.

Hsueh, C.C., Chen, B. Y. and Yen, C. Y. (2009) Understanding effects of chemical structure on azo dye decolorization characteristics by *Aeromonas hydrophila*. *Journal of Hazardous Materials* 167(1-3): 995-1001.

IARC (International Agency for Research on Cancer) (1999) Some chemicals that cause tumors of the kidney or urinary bladder in rodents and some other substances. In: *ortho-Anisidine: IARC Monographs on the Evaluation of Carcinogenic Risk to Humans*, Vol 73. France, World Health Organisation, IARC press. pp 49-58.

Ibrahim, M. S., Ali, H. I., Taylor, K. E., Biswas, J. K. and Bewtra, J. K. (2001) Enzyme-catalyzed removal of phenol from refinery wastewater: feasibility studies. *Water Environment Research* 73(2): 165-172.

Jenkins, F. P., Robinson, J. A., Gellatly, J. B. M. and Salmond, G. W. A. (1972) The no-effect dose of aniline in human subjects and a comparison of aniline toxicity in man and the rat. *Food and Cosmetics Toxicology* 10(5): 671-679.

Karim, Z., and Husain, Q. (2009) Redox-mediated oxidation and removal of aromatic amines from polluted water by partially purified bitter gourd (*Momardica charantia*) peroxidase. *International Biodeterioration and Biodegradation* 63: 587-593.

Kennedy, K., Alemany, K. and Warith, M. (2002) Optimisation of soybean peroxidase treatment of 2,4-dichlorophenol. *Water SA* 28(2): 149-158.

Kinsley, C. and Nicell, J. A. (2000) Treatment of aqueous phenol with soybean peroxidase in the presence of polyethylene glycol. *Bioresource Technology* 73(2): 139-146.

Klibanov, A. M., Tu, T. M. and Scott, K. P. (1983) Peroxidase catalyzed removal of phenols from coal-conversion wastewater. *Science* 221: 259-261.

Klibanov, A. M. (1982) Enzymatic removal of hazardous pollutants from industrial aqueous effluents. *Enzyme Engineering* 6: 319-323.

Klibanov, A. M., and Morris, E. D. (1981) Horseradish peroxidase for the removal of carcinogenic aromatic amines from water. *Enzyme and Microbial Technology* 3(2): 119-122.

Klibanov A. M., Alberti, B. N., Morris, E. D., and Felshin, L. M. (1980) Enzymatic removal of toxic phenols and anilines from waste waters. *Journal of applied biochemistry* 2: 414-421.

Knuston, K., Kirzan, S. and Ragauskas, A. (2005) Enzymatic biobleaching of two recalcitrant paper dyes with horseradish and soybean peroxidase. *Biotechnology Letters* 27(11): 753-758.

Kulla, H. G., Klausener, F., Meyer, U., Ludeke, B. and Leisinger, T. (1983) Interference of aromatic sulfo groups in the microbial degradation of the azo dyes Orange I and Orange II. *Archives of Microbiology* 135(1): 1-7.

Laurenti, E., Ghibaudi, E., Ardisson, S. and Ferrari, R. P. (2003) Oxidation of 2,4-dichlorophenol catalyzed by horseradish peroxidase: characterization of the reaction mechanism by UV-visible spectroscopy and mass spectrometry. *Journal of Inorganic Biochemistry* 95(2-3): 171-176.

Lavine, B. K., Auslander, G. and Ritter, J. (2001) Polarographic studies of zero valent iron as a reductant for remediation of nitroaromatics in the environment. *Microchemical Journal* 70(2): 69-83

Maguire, R. J. and Tkacz, R. J. (1991) Occurrence of dyes in the Yamaska River, Quebec. *Water Pollution Research Journal of Canada*. 26(2): 145-161.

- Mantha, R. (2001) Continuous flow system for combined chemical and enzyme-catalyzed removal of nitroaromatics from synthetic wastewater. Ph.D. Dissertation. University of Windsor, Windsor, Ontario, Canada.
- Mao, L., Luo, S., Huang, Q. and Lu, J. (2013) Horseradish peroxidase inactivation: heme destruction and influence of polyethylene glycol. *Scientific Reports* 3, article 3126. online: <http://www.nature.com/srep/2013/131104/srep03126/full/srep03126.html> accessed: Mar. 17, 2014.
- Marchis, T., Avetta, P., Bianco-Prevot, A., Fabbri D., Viscardi G. and Laurenti, E. (2011) Oxidative degradation of Remazol Turquoise Blue G 133 by soybean peroxidase. *Journal of Inorganic Biochemistry* 105(2): 321-327.
- Market Watch (2013) Colorants market- dyes(reactive, disperse, acid, direct, basic, vat), organic pigments(azo, phthalocyanines, high performance) & inorganic pigments(TiO₂, iron oxide, carbon black & others)- global trends & forecast 2018. Market Watch. PR Newswire. <http://www.marketwatch.com/story/colorants-market-dyes-reactive-disperse-acid-direct-basic-vat-organic-pigments-azo-phthalocyanines-high-performance-inorganic-pigments-tio2-iron-oxide-carbon-black-others-global-trends-forecast-to-2018-2013-08-12>. Last accessed March 6, 2013.
- Matheson, L. J. and Tratnyek, P. G. (1994) Reductive Dehalogenation of chlorinated methanes by iron metal. *Environmental Science and Technology* 28(12): 2045-2053.
- McEldoon, J. P. and Dordick, J. S. (1996) Unusual thermal stability of soybean peroxidase. *Biotechnology Progress* 12(4): 555-558.
- McLean, S., Starmer, G. A. and Thoma, J. (1969) Methaemoglobin formation by aromatic amines. *Journal of Pharmacy and Pharmacology* 21(3): 441-450.
- Modaressi, K., Taylor, K. E., Bewtra, J. K. and Biswas, N. (2005) Laccase-catalyzed removal of bisphenol-A from water: protective effect of PEG on enzyme activity. *Water Research* 39(18): 4309-4316.
- Mohan, S. V., Prasad, K. K., Rao, C. N., and Sarma, P. N. (2005) Acid azo dye degradation by free and immobilized horseradish peroxidase (HRP) catalyzed process. *Chemosphere* 58(8): 1097-1105.
- Morris, P. J. T. and Travis, A. S. (1992) A history of the international dyestuff industry. *American Dyestuff Reporter* 81(11): 59-100.
- Munnecke, D. M. (1976) Enzymatic hydrolysis of organophosphate insecticides, a possible pesticide disposal method. *Applied and Environmental Microbiology* 32(1): 7-13.
- Nakamoto, S., and Machida, N. (1992) Phenol removal from aqueous solutions by peroxidase-catalyzed reaction using additives. *Water Research* 26(1): 49-54.
- Nam, S. and Tratnyek, P. G. (2000) Reduction of azo dyes with zero-valent iron. *Water Research* 34(6): 1837-1845.

Nawar, S. S. and Doma, H. S. (1989) Removal of azo dyes from effluents using low-cost agricultural by-products. *The Science of the Total Environment* 79(3): 271-279.

Nicell, J. A. (2003) Enzymatic treatment of waters and wastes. In: *Chemical Degradation Methods for Wastes and Pollutants: Environmental and Industrial Applications*, Tarr, M. A. (Ed) USA, CRC Press, pp. 384-428.

Nicell, J. A. (1994) Kinetics of Horseradish peroxidase-catalysed polymerization and precipitation of aqueous 4-chlorophenol. *Journal of Chemical Technology Biotechnology* 60(2): 203-215.

Nicell, J. A., Al-Kassim, L., Bewtra, J. K. and Taylor, K. E. (1993) Wastewater treatment by enzyme catalysed polymerization and precipitation. *Biodeterioration Abstracts* 7(1): 1-8.

Nicell, J. A., Saadi, W. and Buchanan I. D. Phenol polymerization by horseradish peroxidase enzyme and an additive. *Bioresource Technology* 54(1): 5-16.

NPRI (2012) National pollutant release inventory online data search. Environment Canada. Released October 11, 2013. <http://www.ec.gc.ca/inrp-npri/donnees-data/index.cfm> then select Aniline.

NTP (National Toxicology Program) (2011) Report on Carcinogens, 12th edition. U.S. Department of Health and Human Services: Public Health Service: NTP. pp.43-45

Ollikka, P., Alhonmaki, K., Leppanen, V., Glumoff, T., Rajjola, T. and Suominen, I. (1993) Decolorization of azo, triphenyl methane, heterocyclic and polymeric dyes by lignin peroxidase isoenzymes from *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 59(12): 4010-4016.

Pakshirajan, K., and Singh, S. (2010) Decolorization of synthetic wastewater containing azo dyes in a batch-operated rotating biological contactor reactor with immobilized fungus. *Industrial & Engineering Chemistry Research* 49(16): 7484-7487.

Palmer, T. (1991) *Understanding Enzymes*, 3rd edition. New York, Ellis Horwood.

Pasti-Grigsby, M. B., Paszczynski, A., Goszczynski, S., Crawford, D. L., and Crawford, R. L. (1992) Influence of aromatic substitution patterns on azo dye degradability by *Streptomyces* spp. and *Phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 58(11): 3605-3613.

Patapas, J., Al-Ansari, M. M., Taylore, K. E., Bewtra, J. K. and Biswas, N. (2007) Removal of dinitrotoluenes from water via reduction with iron and peroxidase-catalyzed oxidative polymerization: a comparison between *Arthromyces ramosus* peroxidase and soybean peroxidase. *Chemosphere* 67(8): 1485-1491.

Pinheiro, H. M., Touraud, E., and Thomas, O. (2004) Aromatic amines from azo dye reduction: status review with emphasis on direct uv spectrophotometric detection in textile industry wastewaters. *Dyes and Pigments* 61(2): 121-139.

Puneter and Page (2004) European ban on certain azo dyes. TFL. <http://www.tfl.com/web/files/eubanazodyes.pdf>. updated Jan 5, 2004. Accessed Mar 3, 2014.

Reardon, E. J. (1995) Anaerobic corrosion of granular iron: measurement and interpretation of hydrogen evolution rates. *Environmental Science and Technology* 29(12): 2936-2945.

Regalado, C., Garcia-Almendarez, B. E., and Duarte-Vazquez, M. A. (2004) Biotechnological applications of peroxidases. *Phytochemistry Reviews* 3(1-2): 243-256.

Reynolds, G. W., Hoff, J. T. and Gillham, R. W. (1990) Sampling bias caused by materials used to monitor halocarbons in groundwater. *Environmental Science and Technology* 24(1): 135-142.

Robinson T., McMullan, G., Marchant, R., and Nigam, P. (2001) Remediation of dyes in textile effluent: a critical review on current treatment technologies with a proposed alternative. *Bioresource Technology* 77(3): 247-255.

Robinson, T., Chandran, B., and Nigam, P. (2002) Removal of dyes from a synthetic textile dye effluent by biosorption on apple pomace and wheat straw. *Water Research* 36(11): 2824-2830.

Rodriquez Couto, S., and Toca- Herrera, J. L. (1996) Lacasses in textile industry. *Biotechnology and Molecular Biology Review* 1(4): 114-120.

Ryan, B. J., Carolan, N. and O'Fagain, C. (2006) Horseradish and soybean peroxidases: comparable tools for alternative niches. *Trends in Biotechnology* 24(8): 355-363.

Saha, B., Taylor K. E., Bewtra, J. K., and Biswas, N. (2008) Laccase-catalyzed removal of diphenylamine from synthetic wastewater. *Water Environment Research* 80(11): 2118-2124.

Sakurai, A., Masuda, M. and Sakakibara (2003) Effect of surfactants on phenol removal by the method of polymerization and precipitation catalysed by *Coprinus cneureus* peroxidase. *Journal of Chemical Technology and Biotechnology* 78(9): 952-958.

Sanchez, S., and Damain, A. L. (2011) Enzymes and bioconversions of industrial, pharmaceutical, and biotechnological significance. *Organic Process Research & Development* 15(1): 224-230.

Sayles, G. D., You, G., Wang, M. and Kupferle, M. J. (1997) DDT, DDD, and DDE dechlorination by zero-valent iron. *Environmental Science and Technology* 31(12): 3448-3454.

Shaffiqu, T. S., Roy, J.J., Nair, R. A., and Abraham, T. E. (2002) Degradation of textile dyes mediated by plant peroxidases. *Applied Biochemistry and Biotechnology* 102-103(1-6): 315-326.

Shan, J., Han, L., Bai, F. and Cao, S. (2003) Enzymatic polymerization of aniline and phenol derivatives catalyzed by horseradish peroxidase in dioxane(II). *Polymers for Advanced Technologies* 14(3-5): 330-336.

Slein, M. W. and Sansone E. B. (1980) *Degradation of Chemical Carcinogens*. Van Nostrand Reinhold, New York.

Spadaro, J. T., Gold, M. H., and Renganthan, V. (1992) Degradation of azo dyes by the lignin-degrading fungus *phanerochaete chrysosporium*. *Applied and Environmental Microbiology* 58(8): 2397-2401.

Spadaro, J. T. (1994) Biological and chemical degradation of azo dyes under aerobic conditions. Ph.D. dissertation. Oregon Graduate Institute of Science and Technology, Hillsboro, Oregon, U.S.A.

Steevenz, A. (2008) Laccase-catalyzed removal of various aromatic compounds from synthetic and refinery wastewater. Ph.D. dissertation. University of Windsor, Windsor, Ontario, Canada.

Steevensz, A., Madur, S., Fend, W., Taylor, K. E., Bewtra, J. K. and Biswas, N. (2014) Crude soybean hull peroxidase treatment of phenol in synthetic and real wastewater: enzyme economy enhanced by Triton X-100. *Enzyme and Microbial Technology* 55(2): 65-71.

Stiborova, M., Asfaw, B., Frei, E., and Schmeiser, H. H. (1996) Oxidation of azo dyes by peroxidase: additional evidence of a one electron mechanism of oxidation of dimethylaminoazobenzene and Sudan I (Solvent Yellow 14). *Collection of Czechoslovak Chemical Communication* 61(6): 962-972.

Stocker, E. (2002) o-Anisidine: risk reduction: Elaboration of a risk reduction strategy under existing substances regulation (EEC) no 793/93. Vienna, Austria, Umweltbundesamt/Federal Environment Agency.

Stolz, A. (2001) Basic and applied aspects in the microbial degradation of azo dyes. *Applied Microbiology Biotechnology* 56(1-2): 69-80.

Suzuki, T., Timofei, S., Kurunczi, L., Dietze, U, and Schuurman, G. (2001) Correlation of aerobic biodegradability of sulfonated azo dyes with the chemical structure. *Chemosphere* 45(1): 1-9.

Taylor, K. E., Al-Kassim, L., Bewtra, J. K., Biswas, N. and Taylor, J. (1996) Enzyme based wastewater treatment: removal of phenols by oxidative enzymes. In: *Environmental Biotechnology: Principles and Applications*, Moo-Young, M., Anderson, W. A. and Chakrabarty, A. M. (Eds) Kluwer Academic Publishers, Dordrecht, pp. 524-532.

Taylor, K. E., Bewtra, J. K. and Biswas, N. (1998) Enzymatic treatment of phenolic and other aromatic compounds in wastewaters. WEFTEC '98 Water Environment Federation Annual Conference & Exposition Proceedings Vol. 3. Orlando, Fl., pp. 349-360.

Tincher, W. C. (1986) Analysis for acid dyes in the Coosa River Basin. US Operating Committee of the Ecological and Toxicological Association of the Dyestuffs Manufacturing Industry. Final Project Report E-27-605. Washington D.C.

Tonegawa, M., Dec, J. and Bollag, J. M. Use of additives to enhance the removal of phenols from water treated with horseradish and hydrogen peroxide. *Journal of Environmental Quality* 32(4): 1222-1227.

TRI (2012) TRI explorer chemical report. U.S. Environmental Protection Agency. Released November 2012. <http://epa.gov/triexplorer> and select Aniline and *o*-Anisidine.

Trivedi, U. J., Bassi, A. S. and Zhu J. (2006) Investigation of phenol removal using sol-gel/alginate immobilized soybean seed hull peroxidase. *The Canadian Journal of Chemical Engineering* 84(2): 239-247.

Ulson de Souza, S. M. A, G., Forgiarini and Ulson de Souza, A. A. (2007) Toxicity of textile dyes and their degradation by the enzyme horseradish peroxidase (HRP), *Journal of Hazardous Materials* 147(3): 1073-1078.

U.S. EPA (United States Environmental Protection Agency) (1994a) OPPT chemical fact sheet: aniline fact sheet: support document (CAS No. 22-53-3). Pollution Prevention and Toxics. 749-F-95-002a. <http://www.epa.gov/chemfact/anali-sd.pdf>. accessed March 3, 2013. Posted Aug. 15, 2002.

U.S. EPA (United States Environmental Protection Agency) (1994b) OPPT chemical fact sheet: aniline fact sheet (CAS No. 22-53-3). Pollution Prevention and Toxics. 749-F-95-002. <http://www.epa.gov/chemfact/anali-fs.pdf>. Accessed March 3, 2013. Posted Aug. 15, 2002.

U.S. EPA (United States Environmental Protection Agency)(2013) Technology Transfer Network - air toxics web site: Aniline. <http://www.epa.gov/ttnatw01/hlthef/aniline.html>. Last updated October 18, 2013.

U.S. EPA (United States Environmental Protection Agency)(2013) Technology Transfer Network - air toxics web site: *o*-Anisidine. <http://www.epa.gov/ttnatw01/hlthef/methoxya.html>. Last updated October 18, 2013.

van der Zee, F. P., Lettinga, G., and Field, J. A. (2001) Azo dye decolourisation by anaerobic granular sludge. *Chemosphere* 44(5): 1169-1176.

Vandevivere, P. C., Bianchi, R., and Verstraete, W. (1998) Treatment and reuse of wastewater from the textile wet-processing industry: review of emerging technologies. *Journal of Chemical Technology and Biotechnology* 72(4): 289-302.

Veith, W. R. and Venkatasubramanian, K. (1973) Enzyme engineering Part I: The utility of supported enzyme systems. *Chemtech*. November. 2:677-684.

Wagner, M. and Nicell, J. A. (2003) Impact of the presence of solids on peroxidase-catalyzed treatment of aqueous phenol. *Journal of Chemical Technology Biotechnology* 78(6):694-702.

Wang, W., Irawan, A., and Ku, Y. (2008) Photocatalytic degradation of Acid Red 4 using titanium dioxide membrane supported on porous ceramic tube. *Water Research* 42(19): 4725-4732.

Weber, E. J. (1996) Iron-mediated reductive transformations: investigation of reaction mechanism. *Environmental Science and Technology* 30(2): 716-719.

Wilberg, K., Assenhaimer, C. and Rubio, J. (2002) Removal of aqueous phenol catalysed by low purity soybean peroxidase. *Journal of Chemical Technology and Biotechnology* 77(7): 851-857.

Wu, J., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1993) Optimization of the reaction conditions for enzymatic removal of phenol from wastewater in the presence of polyethylene glycol. *Water Research* 27(12): 1701-1706.

Wu, Y., Taylor, K. E., Bewtra, J. K. and Biswas, N. (1997) Comparison of additives in the removal of phenolic compounds by peroxidase-catalyzed polymerization. *Water Research* 31(11): 2699-2704.

Yu, J., Wang, X., and Yue, P. L. (2001) Optimal decolorization and kinetic modeling of synthetic dyes by *Pseudomonas* strains. *Water Research* 35(15): 3579-3586.

Yu, J., Taylor, K. E., Zou, H., Biswas, N. and Bewtra, J. K. (1994) Phenol conversion and dimeric intermediates in horseradish peroxidase-catalyzed phenol removal from water. *Environmental Science & Technology* 28(12): 2154-2160.

APPENDIX A

Pictures

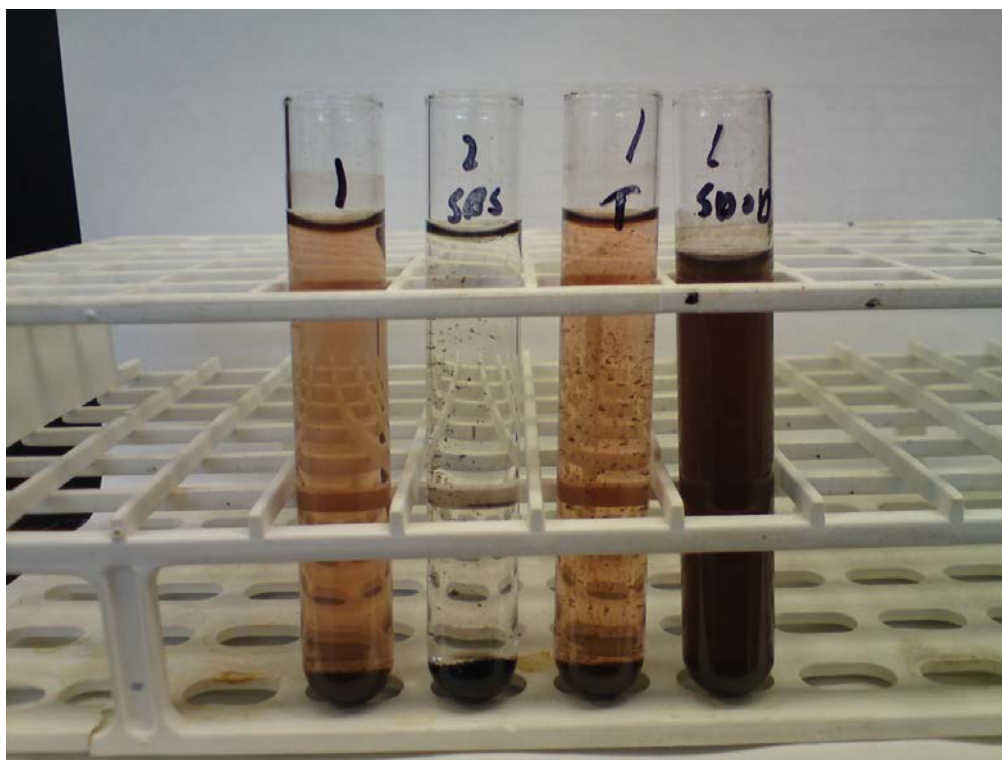


Figure A.1 Treated aniline samples at optimal conditions, no additive, SDS, Triton X-100, and SDOD.

APPENDIX B

Standard Curves

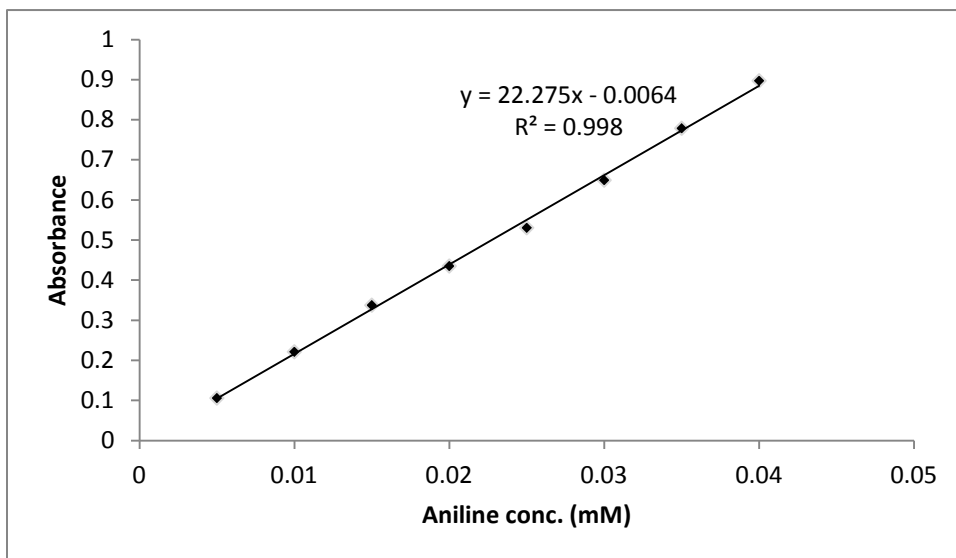


Figure B.1 Aniline TNBS standard curve.

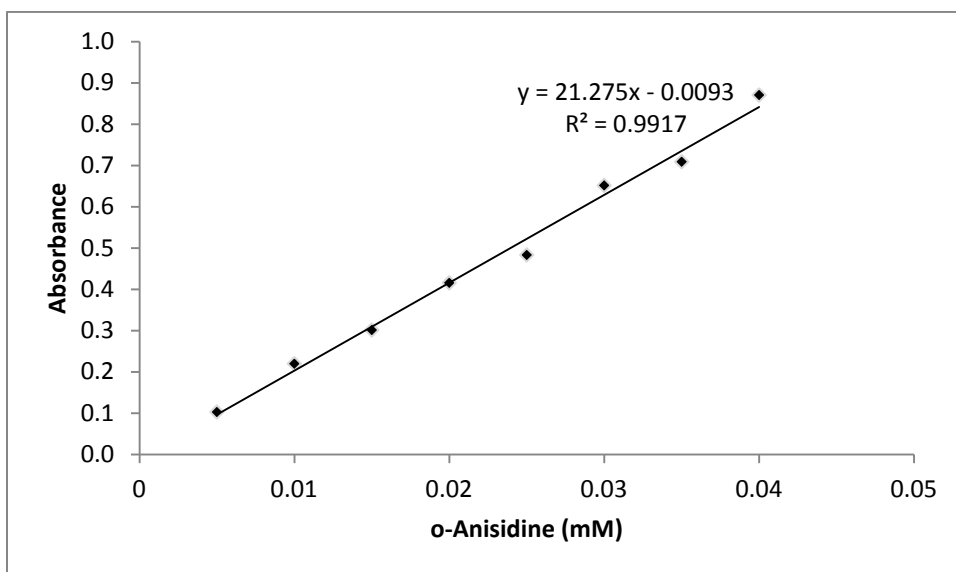


Figure B.2 *o*-Anisidine TNBS standard curve.

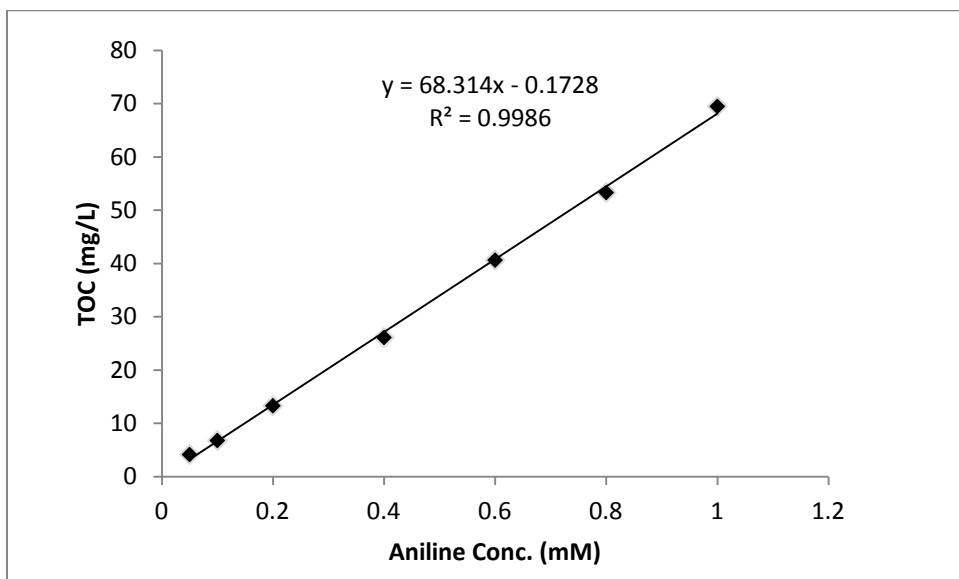


Figure B.3 Aniline TOC standard curve.

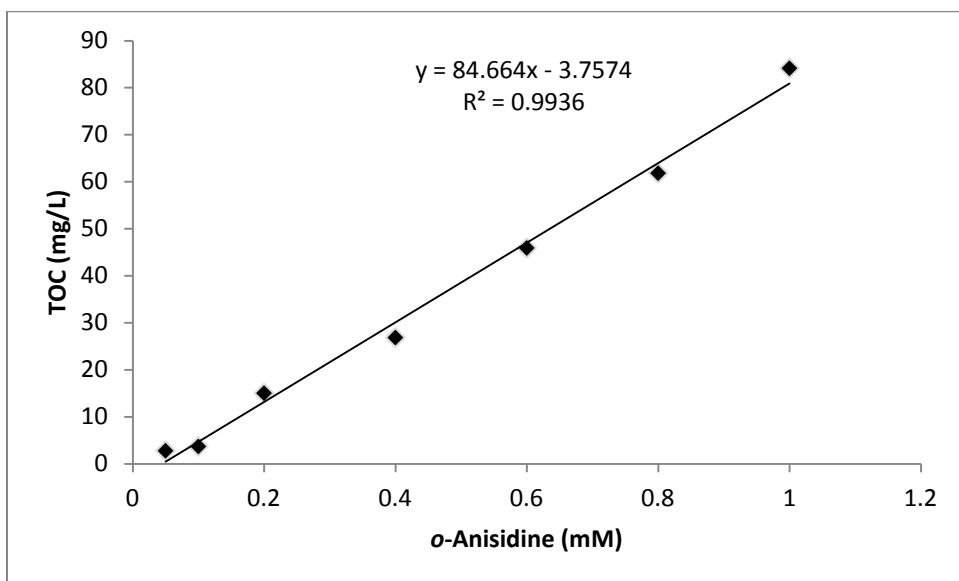


Figure B.4 *o*-Anisidine TOC standard curve.

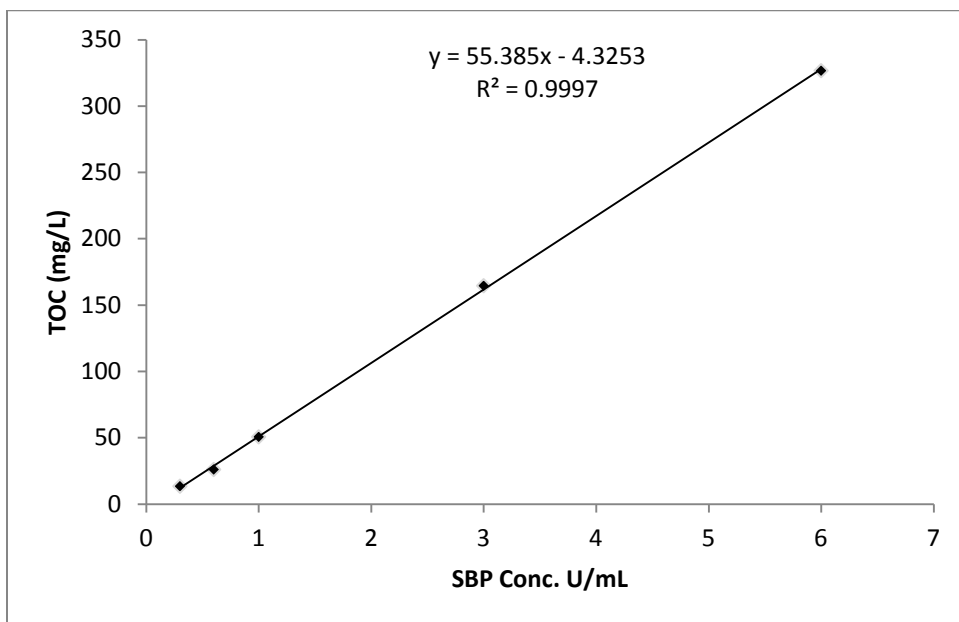


Figure B.5 SBP concentration TOC standard curve.

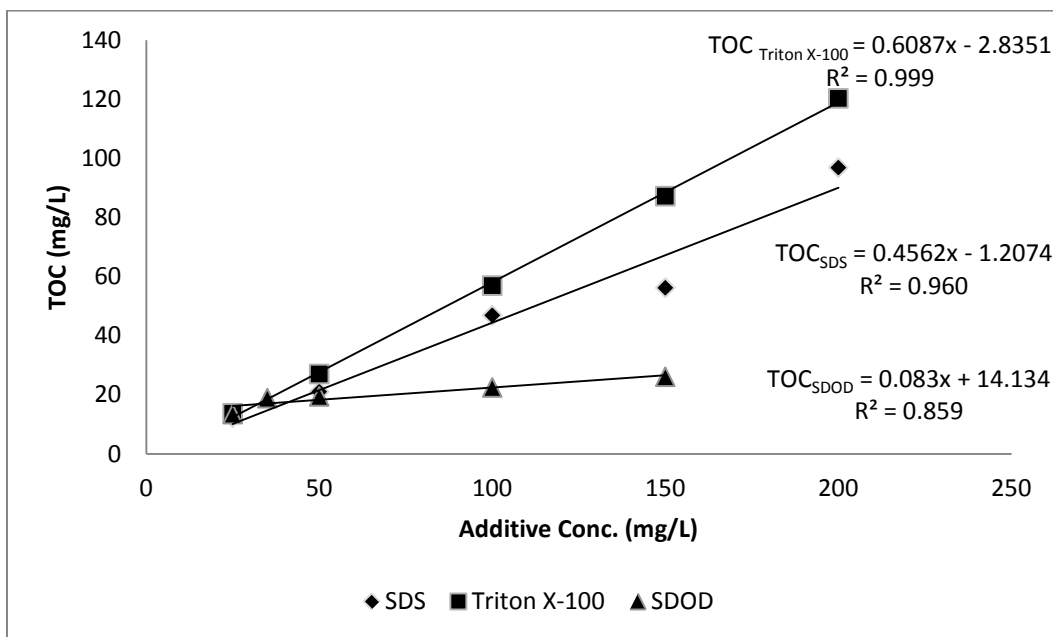


Figure B.6 Additives concentration TOC standard curve.

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